REMARKS

Claims 1-43 and 57-68 constitute the pending claims in the present application, prior to Amendment. Claims 43, 57-60, 62-66, and 68 are currently under consideration as directed to an elected invention and species. Applicants cancel, without prejudice, claims 1-42 which were previously withdrawn from consideration as directed to a nonelected invention. Applicants reserve the right to prosecute claims of similar or differing scope in future applications. Additionally, Application cancel, without prejudice, claims 57, 59, and 62-68. Cancellation of these claims is not in acquiescence to any of the rejections raised during prosecution of the instant application. Claims 57, 59, and 62-63 are cancelled to avoid redundancy in view of Applicants' amendment to claim 43. Claims 64-68 are cancelled to expedite prosecution. Applicants expressly reserve the right to prosecute claims of similar or differing scope.

Claim 43 has been amended to improve the clarity of the claims and to more particularly point out that the compound for use in the claimed method is a "Sonic hedgehog blocking antibody". Support for Applicants' amendment can be found, for example, in paragraphs [0029], [0089], and Example 4 of the published specification.

Claim 58 has been amended to improve the clarity of the claims and to more particularly point out that the Sonic hedgehog blocking antibody inhibits angiogenesis. Support for Applicants' amendment can be found, for example, in paragraph [0042] of the published specification. Applicants' amendment is not believed to narrow the scope of the claim but, rather, is believed to improve the clarity of the claim.

Claims 60 has been amended to improve the clarity of the claims by deleting recitation of "enhanced vascular growth accompanies a solid tumor." Applicants' amendment is not believed to narrow the scope of the claim but, rather, is believed to improve the clarity of the claim. Applicants note that, although claim 61 is directed to a nonelected species, Applicants have made a conforming amendment to claim 61. Applicants' amendment is not believed to narrow the scope of the claim but, rather, is believed to improve the clarity of the claim.

Applicants add new claim 69. Support for the subject matter of claim 69 can be found, for example, in paragraph [0118] of the published specification. No new matter has been entered. Claim 69 reads on the elected invention and species.

Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the Office Action.

Information Disclosure Statement

Applicants note with appreciation that the Information Disclosure Statement of October 22, 2007 has been considered.

Withdrawn Objections and/or Rejections

Applicants note that the objections to the specification have been withdrawn.

Claim Rejection – 35 U.S.C. § 112, first paragraph, enablement

Claims 43, 57-60, 62-66, and 68 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. Applicants traverse this rejection and contend that the rejection is moot in view of the amended claims.

The basis of the rejection appears to be two-fold. First, as detailed on pages 3-5 of the instant Office Action, the Examiner alleges that Applicants' arguments and post-filing date evidence fail to support enablement of the claimed methods, as applied to the elected species of enhanced vascular growth accompanying a solid tumor. The Examiner alleges that the "relevant post-filing date art teaches variability in whether or not (and if so, how) the hedgehog pathway is activated in cancer." See, page 4 of the instant Office Action.

Applicants acknowledge that the post filing date art has revealed differing mechanisms by which hedgehog signaling may be misregulated in various types of cancers. Such mechanisms include genetic alterations in tumor cells that lead to constitutive activation of the hedgehog pathway, as is observed in basal cell carcinoma and medullablastoma, as well as overexpression of hedgehog protein itself in tumors. As such, Applicants acknowledge that methods for modulating hedgehog signaling in cells harboring a mutation in a component of the hedgehog signaling pathway may require different compounds than methods for modulating hedgehog signaling in cells that overexpress hedgehog protein but do not harbor a mutation in a component of the hedgehog signaling pathway.

However, the claimed invention is not directed to modulating hedgehog signaling in tumor cells. Rather, the claimed invention is directed to inhibiting enhanced vascular growth, and the elected species is "enhanced vascular growth accompanying a solid tumor". The claims reflect an alternative approach to treating cancer by inhibiting the vascular growth required to

feed the tumor. Such an approach is independent of whether the tumor itself is hedgehog dependent or hedgehog independent. In fact, since the early 1980s and Judah Folkman's pioneering work on the role of angiogenesis in facilitating tumor growth and metastasis, scientists have been investigating signaling pathways that can be modulated and compounds that can be used to treat cancer by inhibiting vascular growth. The appreciation that inhibition of the blood supply feeding a tumor can be used as a treatment is so generally accepted in the oncology field that the National Cancer Institute's website includes slides intended to explain this concept to the public. Exhibit 1.

The specification and post-filing art support a role for hedgehog signaling in vascular growth. Applicants enclose herewith complete copies of the Pola et al. articles referred to in Applicants' previous reply. Pola et al. (2001) Nature Medicine 7: 706-711 and Pola et al. (2003) Circulation 108: 479-85; enclosed herewith as Exhibits 2-3. Applicants' evidence support the use of compounds that inhibit hedgehog signaling, for example a Sonic hedgehog blocking antibody, in methods for inhibiting vascular growth.

Regardless of whether the specification provides a working example of using a hedgehog compound in the context of a tumor, the pre- and post-filing art is replete with examples whereby angiogenesis is inhibited as part of a cancer treatment methodology. In fact, a search of the clinical trials database maintained by the National Institutes of Health revealed over 1200 results based on the key word search "angiogenesis AND inhibitor AND tumor."

Applicants respectfully submit that the claimed methods, which are based on the use of compounds that inhibit vascular growth, are enabled independently of the cause of the underlying tumor (e.g., whether the underlying tumor is due to a mutation in or misexpression of a component of the hedgehog signaling pathway). The target of the claimed method is vascular growth that accompanies the tumor, and this target is generic to tumors of diverse etiology. Accordingly, Applicants submit that the claims are enabled throughout their scope.

The second basis of the Examiner's rejection, advanced on pages 6-7 of the instant Office Action, is that Applicants have allegedly failed to enable "a method of treatment using a genus of structurally undefined hedgehog signaling antagonists." See, page 6 of the instant Office Action. Applicants respectfully disagree. As detailed is Applicants' previous response, the specification and state of the art support the enablement of the claimed methods. Nevertheless, to expedite prosecution, Applicants have amended claim 43 (and claims depending therefrom) to more

particularly point out that the compound for use in the claimed methods is a Sonic hedgehog blocking antibody. Applicants' amendments are not in acquiescence to any of the rejections raised during prosecution of this application. Applicants reserve the right to prosecute claims of similar or differing scope in this or future applications.

Additionally, Applicants have cancelled claims 57, 59, and 62-68 to avoid redundancy in view of Applicants' amendment to claim 43, as well as to expedite prosecution. Applicants reserve the right to prosecute claims of similar or differing scope in this or future applications. Applicants' cancellation of and amendments to the claims are believed to obviate the rejection.

The use of a Sonic hedgehog blocking antibody to inhibit vascular growth is supported by the working examples provided in the specification, as well as the post-filing date art of, for example, Pola et al. Furthermore, the state of the pre- and post-filing date art of cancer research supports the use of compounds that inhibit vascular growth to treat tumors. Accordingly, Applicants respectfully submit that the claims are enabled throughout their scope. Reconsideration and withdrawal of this rejection are requested.

Claim Rejection – 35 U.S.C. § 112, first paragraph, written description

Claim 43, 57-60, 62-66, and 68 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. Applicants traverse this rejection and contend that the rejection is moot in view of the amended claims.

Applicants reiterate the arguments of record and maintain that the specification provides ample support to evince that Applicants had possession of the claimed invention. In further support of Applicants' previous arguments that the factual situation in this case is distinguishable from the scenarios facing the court in *Vas-Cath*, *Fiers*, and *Amgen*, Applicants note that in *Capon v. Eshhar*, the Federal Circuit discussed in detail that the written description requirement must be analyzed in the context of the particular invention, technology, and state of the art ("the 'written description' requirement states that the patentee must describe the invention, it does not state that every invention must be described in the same way). *Capon v. Eshhar*, 418 F.3d 1349, 1358 (Fed. Cir. 2005). The claims at issue in *Capon* were directed to chimeric DNAs and expression vectors comprising the chimeric DNAs. Although the claims at issue in *Capon* were directed to biological compositions, the court still distinguished the factual situation from that of *Lilly*, *Fiers*, and *Amgen*. The court noted that in *Lilly*, *Fiers*, and *Amgen*, the claimed

compositions included biological material that was unknown and had not been characterized. *Capon*, 418 F.3d at 1357-1358. In contrast, the compositions in *Capon*, although broadly and generically claimed without reference to particular sequences, were based on combining elements for which known examples existed in the art.

The factual situation in the instant case is more analogous to *Capon* than to *Lilly*, *Fiers*, and *Amgen*. Various hedgehog compounds, including nucleic acids, polypeptides, blocking antibodies, and small molecule inhibitors were already known in the art prior to Applicants' invention. Accordingly, Applicants' claims, which describe the use of such compounds, is unlike an attempt to claim an unknown biological material without reference to its specific sequence. In the present case, one of skill in the art can readily envision that which is claimed.

In the paragraph bridging pages 8 and 9, the Examiner provides an example of how *Lilly*, *Fiers*, and *Amgen* would be just as applicable to a method claim as to a composition claim. However, the example provided by the Examiner is one in which the compound itself was previously unknown. In contrast, the instant factual situation is more akin to *Capon*, where the art provided numerous examples of the biological compositions themselves. Accordingly, Applicants maintain that prevailing case law supports Applicants' position that the claims, prior to Amendment, are fully compliant with the written description requirement.

The position articulated in *Capon* was followed by the Federal Circuit in *Falkner v. Inglis*. *Falkner v. Inglis*, 468 F.3d 1357, 1369 (Fed. Cir. 2006) ("As we stated in *Capon*, the 'written description' requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way."). *Falkner* additionally held that

> "(1) examples are not necessary to support the adequacy of a written description; (2) the written description standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure."
> *Falkner*, 467 F.3d at 1367.

The foregoing arguments are equally applicable to claims reciting mean-plus-function language.

Nevertheless, to expedite prosecution, Applicants have amended claim 43 (and claims depending therefrom) to more particularly point out that the compound for use in the claimed methods is a Sonic hedgehog blocking antibody. Support for Applicants' amendment can be found, for example, in paragraphs [0029], [0089], and Example 4 of the published specification. Applicants' amendment is not in acquiescence to the rejection or to any of the arguments advanced by the Examiner during prosecution of the instant application. Applicants expressly reserve the right to prosecute claims of similar or differing scope.

Additionally, to expedite prosecution, Applicants have cancelled claims 57, 59, and 62-68. Cancellation of these claims is not in acquiescence to the rejection or to any of the arguments advanced by the Examiner during prosecution of the instant application. Applicants expressly reserve the right to prosecute claims of similar or differing scope. In view of Applicants' amendments, reconsideration and withdrawal of this rejection is requested. Applicants note that amended claim 43 is directed to the use of a Sonic hedgehog blocking antibody in the claimed methods. The Examiner indicated that claims directed to such subject matter satisfied the written description requirement. See, page 10 of the instant Office Action.

New Rejections

Claim Rejection – 35 U.S.C. § 112, first paragraph, written description (new matter)

Claims 43, 57-60, 62-66, and 68 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement for allegedly containing new matter. Applicants traverse this rejection and contend that the rejection is moot in view of the amended claims.

Claim 43 (and claims depending therefrom) is rejected because the specification allegedly fails to support recitation of "a hedgehog antagonist capable of inhibiting hedgehog signaling." Applicants respectfully disagree with the Examiner's assessment and contend that, at the time of filing, one of skill in the art would have readily recognized that Applicants were in possession of methods for using a broad range of hedgehog compounds to inhibit abnormally enhanced vascular growth.

Nevertheless, to expedite prosecution, Applicants have amended claim 43 to more particularly point out that the compound for use in the claimed methods is a Sonic hedgehog blocking antibody. Support for Applicants' amendment can be found, for example, in paragraphs

[0029], [0089], and Example 4 of the published specification. No new matter has been entered. Applicants note that a Sonic hedgehog blocking antibody (an antibody that binds to Sonic hedgehog protein to block its activity) was specifically used in the examples disclosed in the specification and is exemplary of a compound that inhibits hedgehog signaling.

Applicants' amendment is not in acquiescence to the rejection. Applicants expressly reserve the right to prosecute claims of similar or differing scope. Applicants' amendment is believed to obviate the rejection, and reconsideration and withdrawal of the rejection is requested.

Claim 57 is rejected because the specification allegedly lacks support for the genus of hedgehog antibodies that allegedly encompass antibodies against any component of the hedgehog pathway. Applicants traverse. Applicants note that claims 59, 62, and 63 depend from claim 57 and are rejected on the same basis.

Applicants contend that this rejection is based on the Examiner's unreasonably broad interpretation of the term "hedgehog antibody" as encompassing antibodies other than antibodies that bind to hedgehog protein. Nevertheless, to expedite prosecution and as detailed above, Applicants have amended claim 43 to point out that the compound for use in the claimed method is a Sonic hedgehog blocking antibody (an antibody that binds to Sonic hedgehog protein to block its activity). In view of Applicants' amendment to claim 43, Applicants have cancelled claims 57, 59, 62, and 63 to avoid redundancy. Applicants expressly reserve the right to prosecute claims of similar or differing scope. Applicants' cancellation of claims 57, 59, 62, and 63 render this rejection moot, and withdrawal of this rejection is requested.

Claim 58 is rejected because the specification allegedly fails to support recitation of "a method of inhibiting angiogenesis." Applicants traverse. Applicants note that claims 59 and 65 are rejected on the same basis.

Applicants respectfully disagree with the Examiner's assessment. The specification repeatedly discusses vascular growth and methods for inhibiting vascular growth. Paragraph [0042] defines "vascular growth" as *at least one of* vasculogenesis and angiogenesis. (Emphasis added.). Applicants contend that this definition of vascular growth reflects the possession of modulating either or both vasculogenesis and angiogenesis. Accordingly, Applicants contend that claim 58 is fully supported by the specification.

Nevertheless, to expedite prosecution, Applicants have amended claim 58 to improve the clarity of the claim. Applicants' amendment is not in acquiescence to the rejection. Applicants' amendment is not believed to narrow the scope of the claim but, rather, is intended to improve the clarity of the claim. Applicants reserve the right to prosecute claims employing similar or differing language in this or future applications.

Claims 59 and 65 were rejected on the same grounds. Applicants have cancelled claim 59 to avoid redundancy in view of Applicants' amendment to claim 43. Applicants have cancelled claim 65 to expedite prosecution. Cancellation of claims 59 and 65 renders the rejection moot. Applicants expressly reserve the right to prosecute claims of similar or differing scope. In view of Applicants' amendment to claims 58, and in view of cancellation of claims 59 and 65, reconsideration and withdrawal of this rejection is requested.

Claim 60 is rejected because the specification allegedly fails to support recitation of "the enhanced vascular growth accompanies a tumor." Rather, the Examiner alleges that the specification supports methods for treating excess vascular growth found "in" a tumor. See, page 12 of the instant Office Action. Applicants traverse and contend that the rejection is moot in view of the amended claims. Applicants note that claims 62, 63, 66, and 68 are rejected on the same basis.

This rejection is based on the Examiner's unreasonably broad interpretation of the term "accompanying", as recited in the claims, and unreasonably narrow interpretation of the term "in", as recited in the specification. In contrast to the Examiner's interpretation, Applicants contend that, based on the knowledge in the art and the context of the specification and claims, the reasonable interpretation is that both terms refer to the same type of vascular growth that occurs *in the context of* cancer. For example, Applicants note that the word "in" is used in both paragraphs [0005] and [0118]. Paragraph [0005] describes the abnormal vascular growth observed *in* several conditions including tumors and rheumatoid arthritis. Clearly, the Examiner's narrow interpretation of "in" to refer to vascular growth within a tumor makes no sense when applied to rheumatoid arthritis. Rather, the reasonable interpretation of "in", as used in the specification is "in the context of". Applicants' recitation of "accompanying" was intended to capture this teaching of the specification.

Nevertheless, to expedite prosecution, Applicants have amended claim 60 to improve the clarity of the claims and to point out that, as supported by the specification, the claimed method

is a method for treating a solid tumor. Applicants' amendment is not believed to narrow the scope of the claim but, rather, is intended to improve the clarity of the claim. Although claim 61 is withdrawn from consideration as directed to a nonelected species, Applicants have made a conforming amendment to claim 61. Applicants' amendment to claim 61 is believed to improve the clarity of the claim but is not believed to narrow its scope. Applicants' amendments are not in acquiescence to the rejection. Applicants reserve the right to prosecute claims employing similar or differing language in this or future applications.

Claims 62, 63, 66, and 68 were rejected on the same grounds. Applicants have cancelled claims 62 and 63 to avoid redundancy in view of Applicants' amendment to claim 43. Applicants have cancelled claims 66 and 68 to expedite prosecution. Cancellation of claims 62, 63, 66, and 68 renders the rejection moot. Applicants expressly reserve the right to prosecute claims of similar or differing scope. In view of Applicants' amendments to claims 60 and 61, and in view of cancellation of claims 62, 63, 66, and 68, reconsideration and withdrawal of this rejection is requested.

Claim 64 (and claims depending therefrom) is rejected because the specification allegedly fails to support recitation of "means for inhibiting hedgehog signaling." Applicants respectfully disagree with the Examiner's assessment and contend that, at the time of filing, one of skill in the art would have readily recognized that antagonistic hedgehog compounds include a range of compounds that inhibit hedgehog signaling. Nevertheless, to expedite prosecution, Applicants have cancelled claim 64 (and claims depending therefrom). Cancellation of claims 64-68 is not in acquiescence to the rejection. Applicants expressly reserve the right to prosecute claims of similar or differing scope. Applicants' cancellation of claims 64-68 render the rejection moot, and withdrawal of this rejection is requested.

In view of the foregoing, Applicants request reconsideration and withdrawal of the rejection. The pending claims are fully supported by the specification.

Claim Rejection – 35 U.S.C. § 112, second paragraph

Claims 64-66 and 68 are rejected under 35 U.S.C. 112, second paragraph, for allegedly failing to particularly point out and distinctly claim the subject matter that Applicants regard as the invention. Applicants traverse this rejection.

The basis of the rejection is that the specification allegedly fails to set forth adequate disclosure to support the means plus function language recited in claims 64-68. Applicants respectfully disagree. Nevertheless, to expedite prosecution, Applicants have cancelled claims 64-66 and 68 (as well as claim 67 which was directed to a nonelected species). Cancellation of claims 64-68 is not in acquiescence of the rejection. Applicants expressly reserve the right to prosecute claims of similar or differing scope. Cancellation of claims 64-68 renders the rejection moot, and withdrawal of this rejection is requested.

Related Applications

The following co-pending applications have already been made of record during prosecution of this application: application serial number 10/727,195; application serial number 09/883,848; application serial number 10/652,686; application serial number 09/977,864; and application serial number 10/652,298. Prosecution in the co-pending applications is on going. The most recent action in application serial number 10/727,195 is an after-final response filed February 28, 2008 (responsive to a final Office Action mailed November 9, 2007). The most recent action in application serial number 09/883,848 is a final Office Action mailed March 3, 2008. The most recent action in application serial number 10/652,686 is a response filed January 29, 2008 (responsive to an Office Action mailed November 2, 2007). The most recent action in application serial number 09/977,864 is a final Office Action mailed March 17, 2008. The most recent action in application serial number 10/652,298 is a final Office Action mailed April 4, 2008.

CONCLUSION

If any clarification of the above response would facilitate prosecution of this application, Applicants respectfully request that the Examiner contact the undersigned at 617-951-7000. Should any further extension or other fee be required for timely consideration of this submission, Applicants hereby petition for same and request that the fee be charged to **Deposit Account No. 18-1945, under Order No. HUIP-P02-060**.

Respectfully Submitted,

Date: April 8, 2008

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Understanding Cancer Series: Angiogenesis

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Posted: 01/28/2005 **Reviewed:** 09/01/2006

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New Study of Targeted Therapies for Breast Cancer
The Nation's Investment in Cancer Research FY 2009
President's Cancer Panel Annual Report: 2006-2007
Cancer Trends Progress Report: 2007 Update
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Slide 8 Angiogenesis and Cancer

Before the 1960s, cancer researchers believed that the blood supply reached tumors simply because pre-existing blood vessels dilated. But later experiments showed that angiogenesis--the growth of the new blood vessels--is necessary for cancerous tumors to keep growing and spreading.

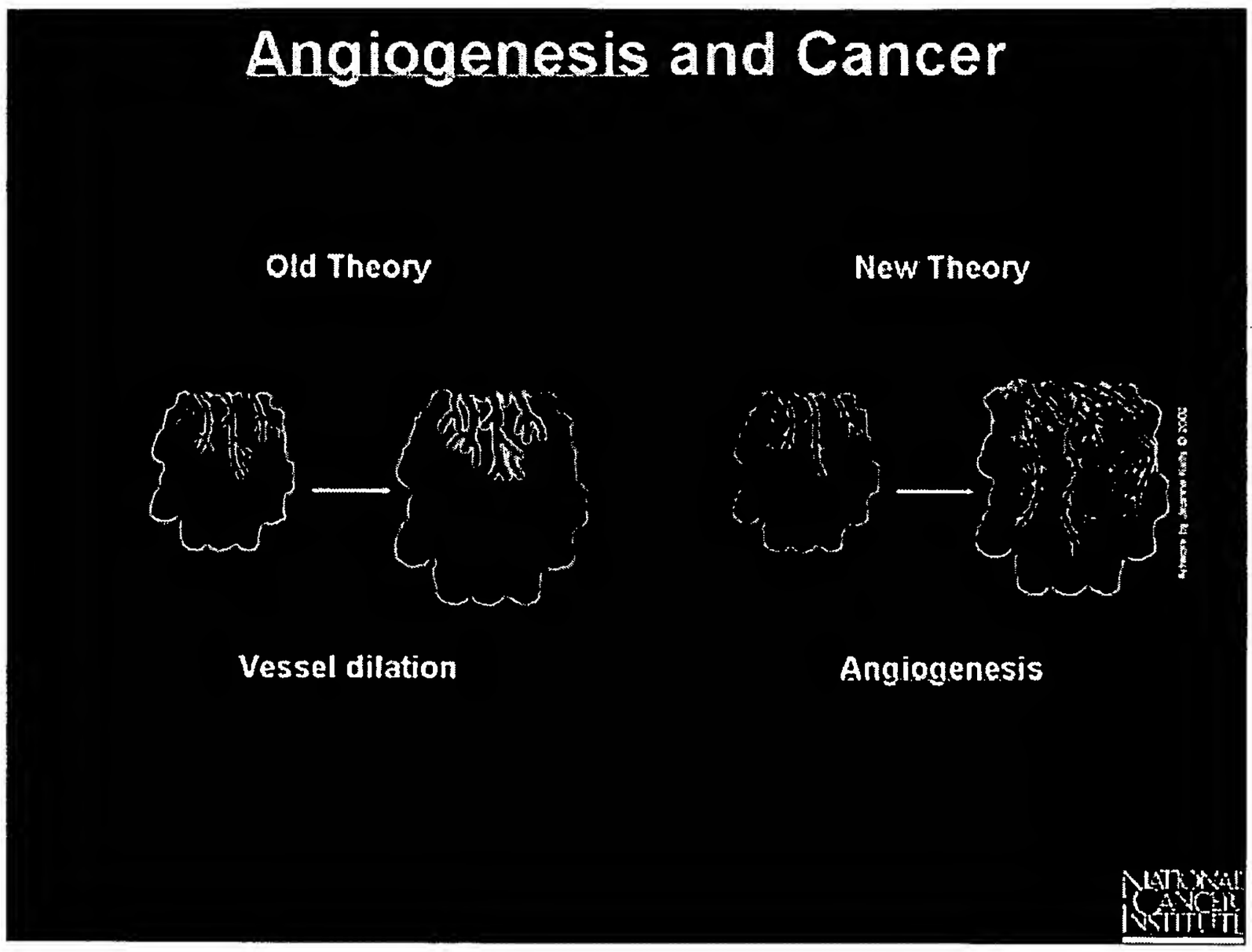

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Exhibit 1

Understanding Cancer Series: Angiogenesis

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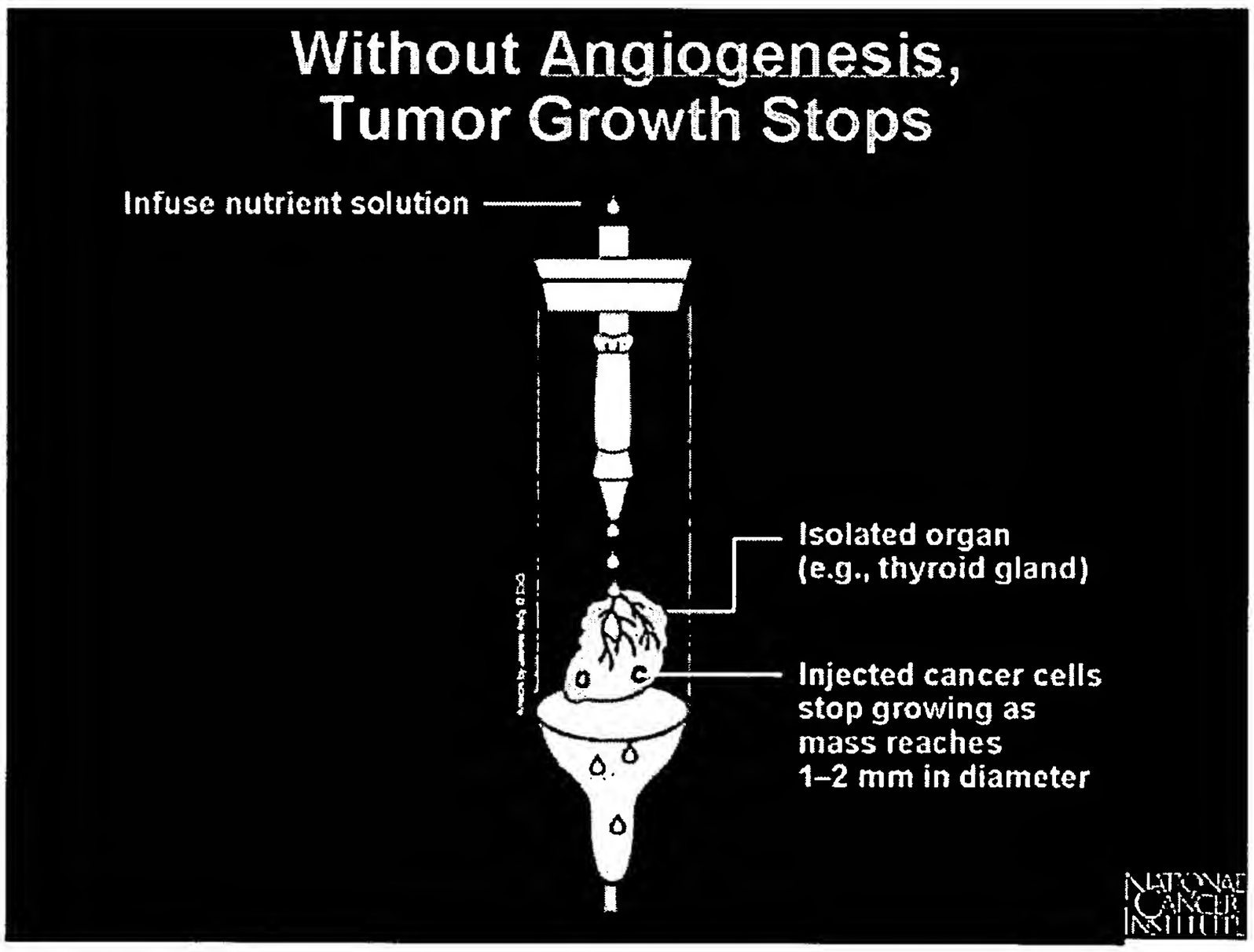
Past Highlights

Slide 9 Without Angiogenesis, Tumor Growth Stops

In early experiments, researchers asked whether cancer growth requires angiogenesis. Scientists removed a cancerous tumor from a laboratory animal and injected some of the cancer cells into a normal organ removed from the same strain of animal. The organ was then placed in a glass chamber and a nutrient solution was pumped into the organ to keep it alive for a week or two. Scientists found that the cancer cells grew into tiny tumors but failed to link up to the organ's blood vessels. As a result, tumor growth stopped at a diameter of about 1-2mm. Without angiogenesis, tumor growth stopped.



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The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors

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Sonic hedgehog (Shh) is a prototypical morphogen known to regulate epithelial/mesenchymal interactions during embryonic development. We found that the hedgehog-signaling pathway is present in adult cardiovascular tissues and can be activated *in vivo*. Shh was able to induce robust angiogenesis, characterized by distinct large-diameter vessels. Shh also augmented blood-flow recovery and limb salvage following operatively induced hind-limb ischemia in aged mice. *In vitro*, Shh had no effect on endothelial-cell migration or proliferation; instead, it induced expression of two families of angiogenic cytokines, including all three vascular endothelial growth factor-1 isoforms and angiopoietins-1 and -2 from interstitial mesenchymal cells. These findings reveal a novel role for Shh as an indirect angiogenic factor regulating expression of multiple angiogenic cytokines and indicate that Shh might have potential therapeutic use for ischemic disorders.

Hedgehog (Hh) proteins act as morphogens in many tissues during embryonic development[1-8]. The mature forms of Hh are 19-kD proteins that interact with heparin through an N-terminal basic domain and are tethered to the cell membrane through cholesterol and fatty acyl modification[9-11]. Hh acts upon mesoderm in epithelial–mesenchymal interactions that are crucial to the formation of limb, lung, gut, hair follicles and bone[2-6]. Among the three highly conserved mammalian Hh genes, Sonic hedgehog (Shh) is the most widely expressed during development[12,13] and Shh deficiency in mice is embryonically lethal leading to multiple defects beginning in early to mid gestation[1,3-5]. Indian hedgehog (Ihh) is less widely expressed and Ihh-deficient mice survive to late gestation with skeletal and gut defects[4,6,13]. Desert hedgehog (Dhh) is expressed in the peripheral nerves, male gonads, as well as the endothelium of large vessels during development[13]. Dhh-deficient mice are viable but have peripheral-nerve and male-fertility defects[7,8].

Hh signaling occurs through the interaction of Hh protein with its receptor, Patched-1 (Ptc1 encoded by *Ptch*)[14]. This leads to activation of a transcription factor, Gli, which induces expression of downstream target genes including *Ptch* and *Gli* themselves[15-23]. Thus Ptc1 and Gli are both required components as well as transcriptionally induced targets of the Hh signaling pathway.

Several recent observations point to the involvement of Hh in vascularizing certain embryonic tissues. First, hypervascularization of neuroectoderm is seen following transgenic overexpression of Shh in the dorsal neural tube[24]. Second, Shh-deficient zebrafish exhibits disorganization of endothelial precursors and an inability to form the dorsal aorta or axial vein[25]. Third, Shh-deficient mice lack proper vascularization of the developing lung[3]. Fourth, Ihh, expressed by prehypertrophic chondrocytes, regulates the rate of chondrocyte maturation, a process closely correlated to the induction of angiogenesis in bone[26,27]. Finally, the induction of anagen in the hair follicle requires both Shh and angiogenesis[28,29]. Although these findings implicate the Hh pathway in vascular development, it is not clear whether these effects are due to a direct angiogenic action of Hh.

Here, we used postnatal mouse models to directly test the impact of Shh on vascularization *in vivo*. We show that cells in the adult cardiac and vascular tissues express Ptc1 and can respond to exogenous Hh by Ptc1 overexpression. In addition, we tested the angiogenic properties of Shh in the corneal and ischemic hind-limb models of angiogenesis. We found that Shh is a potent angiogenic factor, and when administered to aged mice it is able to induce robust neovascularization of ischemic hind-limbs. Shh-induced angiogenesis is characterized by large-diameter vessels. Investigation of the mechanism responsible for these findings established that Shh is an indirect angiogenic agent, inducing upregulation of two families of angiogenic growth factors, including vascular endothelial growth factor (VEGF) and the angiopoietins Ang-1 and Ang-2. Our data indicate a novel and unexpected biological activity for Hh with potential therapeutic implications.

Hh signaling in postnatal vasculature

In juvenile and adult mice, we found that Ptc1 is normally expressed in cardiovascular tissues (Fig. 1). We visualized Ptc1 expression using β-galactosidase (β-gal) staining of vascular tissues from mice that have a non-disruptive insertion of a nuclear localization signal (NLS)-tagged *lacZ* reporter gene upstream of the *Ptch* coding region (NLS-*Ptch-lacZ* mice). Ptc1 expression corresponds to *lacZ* expression in postnatal tissues and does not appear to be altered by *lacZ* insertion (L. Ling, unpublished observations). When examined for nuclear β-gal expression, NLS-*Ptch-lacZ* mice exhibited basal Ptc1 expression in adventitial cells,

Exhibit 2

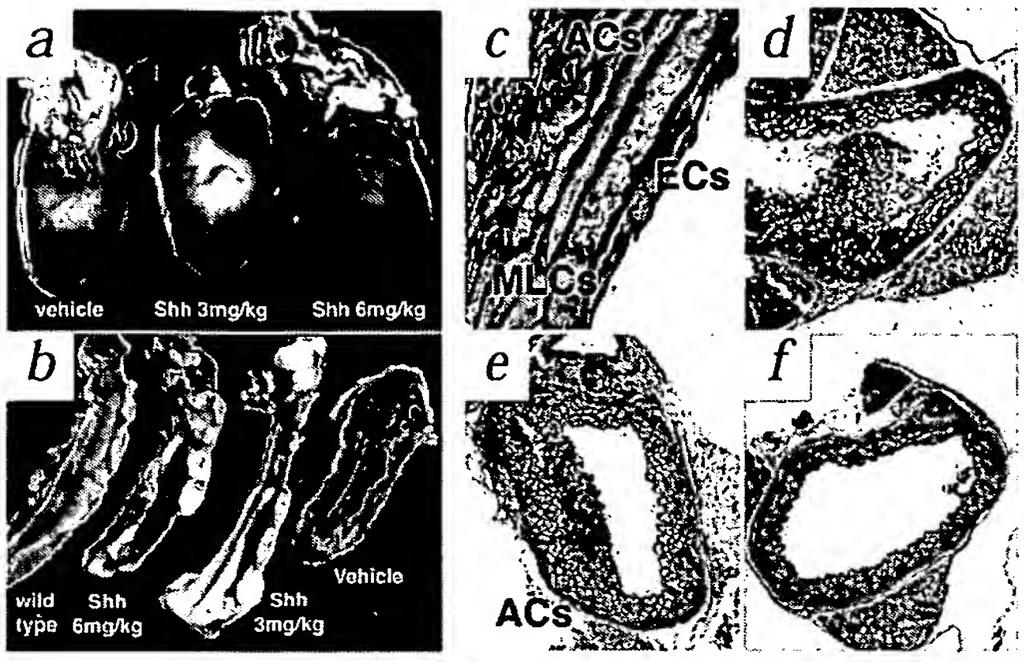


Fig. 1 *Ptch* expression and activation in postnatal cardiovascular tissues. ***a*** and ***b***, Hearts (*a*) and aortas (*b*) from NLS-*Ptch-lacZ*. Vehicle-treated mice exhibit a basal level of *Ptch* expression; administration of Shh result in a dose-dependent increase in *Ptch* expression in both hearts and aortas. ***e*** and ***d***, Paraffin cross sections from vehicle-treated mice (*c*) or untreated (*d*) mice show *Ptch* expression in endothelial cells (ECs), medial layer cells (MLCs) and adventitial cells (ACs). ***e*** and ***f***, Treatment with Shh (*e*) increases *Ptch* expression in adventitial cells. Aortas from wild-type littermates treated with Shh show no *Ptch* expression (*f* and *b*). Magnification ***c***, x200; ***d-f***, x100.

endothelial cells and cells in the medial layer of the vasculature (Fig. 1*c*). These results indicated that adult cardiovascular tissues have several resident populations of cells that might be responsive to Hh. To test this hypothesis, day 6 postnatal NLS-*Ptch-lacZ* mice were injected subcutaneously with Shh once daily for three days. This treatment induced a dose-dependent increase in Ptc1 expression in coronary arteries and aortas (Fig. 1*a*, *b* and *e*). In particular, adventitial cells showed a significant increase in Ptc1 expression (Fig. 1*e*). These cells were vimentin-positive, consistent with aortic adventitial fibroblasts (data not shown).

Shh induces vascular growth and promotes limb salvage

We tested the potential for Shh to act upon the adult vasculature and protect against ischemic injury by administering Shh, the VEGF-1 isoform recombinant human (rh)$VEGF_{165}$ or control to aged mice undergoing unilateral, surgically induced hind-limb ischemia. Aged mice have impaired angiogenesis, decreased blood-flow recovery, and typically develop limb necrosis from ischemic injury due to an inherent compromise in endogenous neovascularization[30]. A blinded evaluation showed that two-year-old mice receiving control developed profound consequences of hind-limb ischemia (including auto-amputation and foot/leg necrosis): 65% at day 7 after surgery, 73% at day 14, 80% at day 21, and 82% at day 28 (Fig. 2*a*). Similarly, mice treated with intramuscular injections of $rhVEGF_{165}$ had severe necrosis or auto-amputation of the ischemic limb comparable to vehicle-treated mice (data not shown). In contrast, we observed a sharp increase in limb salvage in mice treated with Shh. In this group, the percentage of auto-amputated limbs and foot/leg necrosis decreased to 25% at day 7 after surgery, 47% at day 14, 50% at day 21, and 50% at day 28 (Fig. 2*a*). Complete limb salvage after 21 and 28 days follow-up was obtained in half of the mice treated with Shh compared with less than 20% in the vehicle and $rhVEGF_{165}$-treated groups.

Laser power doppler imaging (LDPI) performed independently by two blinded operators demonstrated a progressive increase in the blood flow of ischemic hind limbs in Shh-treated mice, with significant differences seen at day 28 ($P < 0.01$) (Fig. 2*b*). In contrast, we observed no significant increase in hind-limb perfusion beyond 28 days of follow-up in control mice. At day 28 after surgery, the Doppler flow ratio was significantly increased in Shh-treated mice in comparison to the groups treated with $rhVEGF_{165}$ or vehicle ($P < 0.05$) (Fig. 2*b* and data not shown).

Likewise, capillary density at day 28 after surgery was significantly increased in Shh-treated versus $rhVEGF_{165}$- and control-treated mice ($P < 0.001$ and $P < 0.0001$, respectively) (Fig. 2*c*, *d* and data not shown). Neovascularization induced by Shh was characterized not only by increased numbers of capillaries, but also by a substantial increase in vessel diameter (Fig. 2*d*).

Shh-induced angiogenesis has distinctive morphology

To determine the basis of augmented neovascularization in response to Shh, we used the murine corneal angiogenesis model. We implanted pellets containing Shh and/or VEGF, or control in the corneas of 8–12-week-old C57BL/6J mice. Six days after implantation, both VEGF- and Shh-treated eyes exhibited growth of neovessels whereas none induced by control pellets (Fig. 3*a*, *b* and *c*). Whole-mount fluorescent BS1 lectin (Bandeiraea simplicifolia lectin-1, an endothelial cell marker) staining and CD31 immunostaining of cross sections showed several striking differences in morphology between Shh-induced neovessels and those induced by VEGF. Consistent with the previous observations in the is-

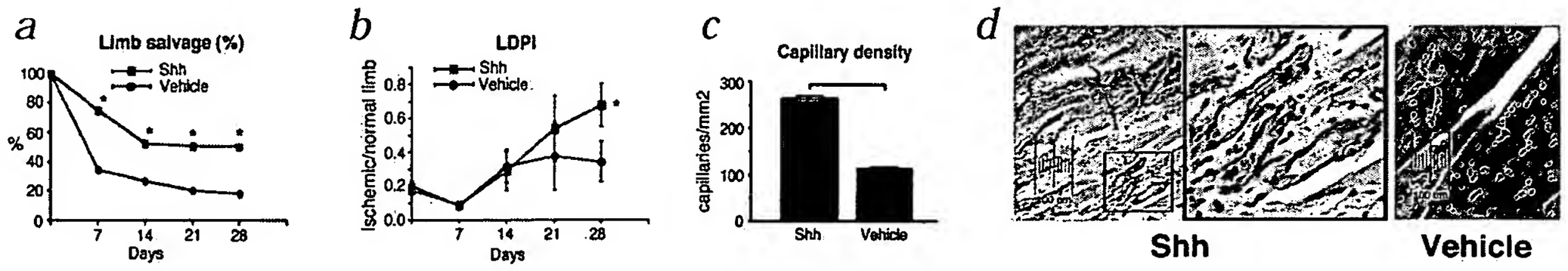


Fig. 2 Shh increases limb salvage, blood flow and capillary density in the setting of ischemia. ***a***, Limb salvage: at each time point, the percentage of limb salvage is statistically significantly higher in Shh-treated group (■) compared with vehicle (●). *, $P < 0.05$. ***b***, Blood flow: ischemic/normal leg perfusion ratio is extremely low in both groups immediately after surgery, but progressively increases over time in Shh-treated mice (■), achieving significant improvement by day 28. *, $P < 0.01$. In contrast, no increase in hind-limb perfusion was seen over time in mice treated with vehicle (●). Ischemic/normal leg perfusion ratio at day 28 is significantly higher in Shh-treated mice compared with vehicle (0.681 ± 0.126 versus 0.344 ± 0.119; $P < 0.05$). ***c***, Capillary density at day 28 after surgery is significantly increased in mice treated with Shh compared with vehicle ($P < 0.0001$). ***d***, Representative pictures of capillary density show that the number of vessels is increased in Shh-treated tissues. A higher magnification (×400) of Shh-treated skeletal muscle (middle) shows a substantial increase in vessel diameter.

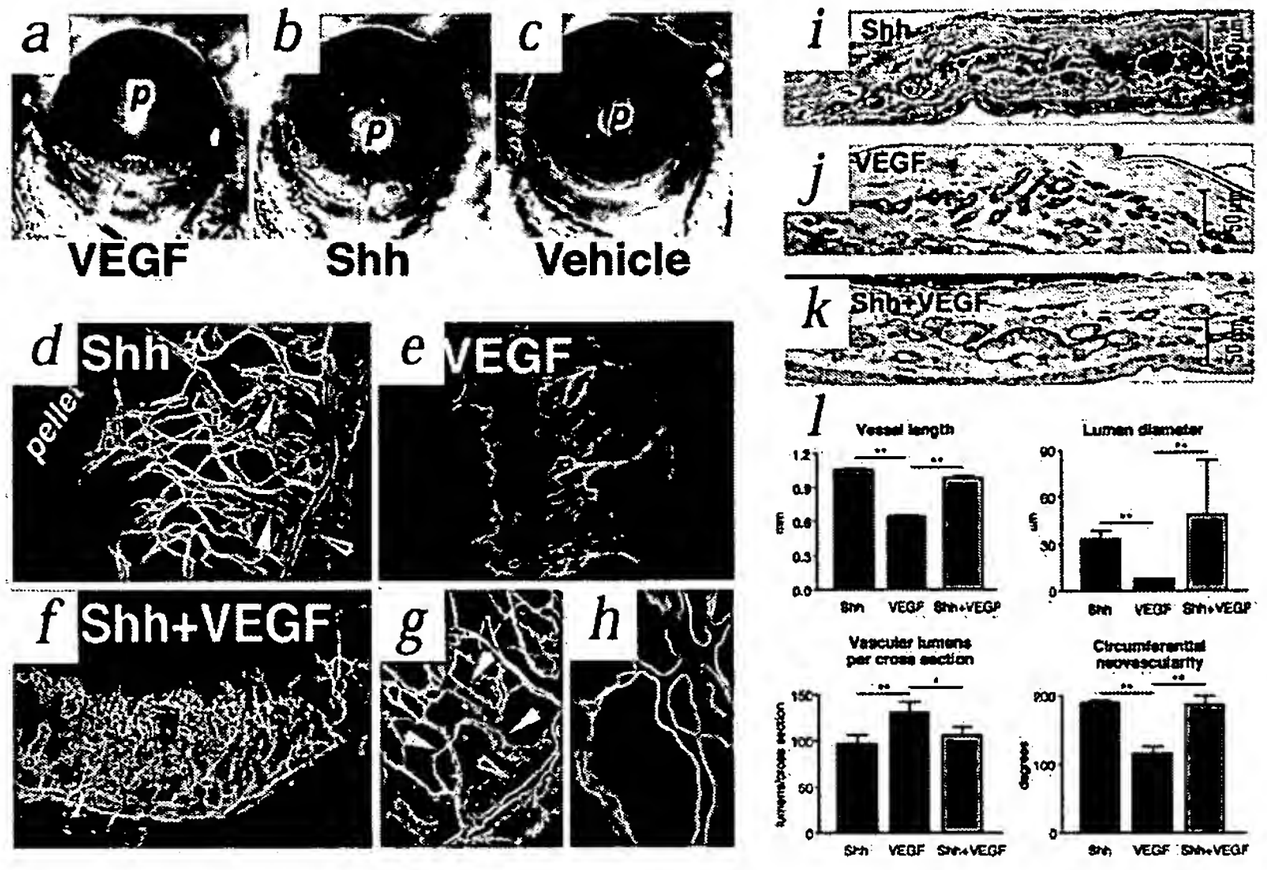
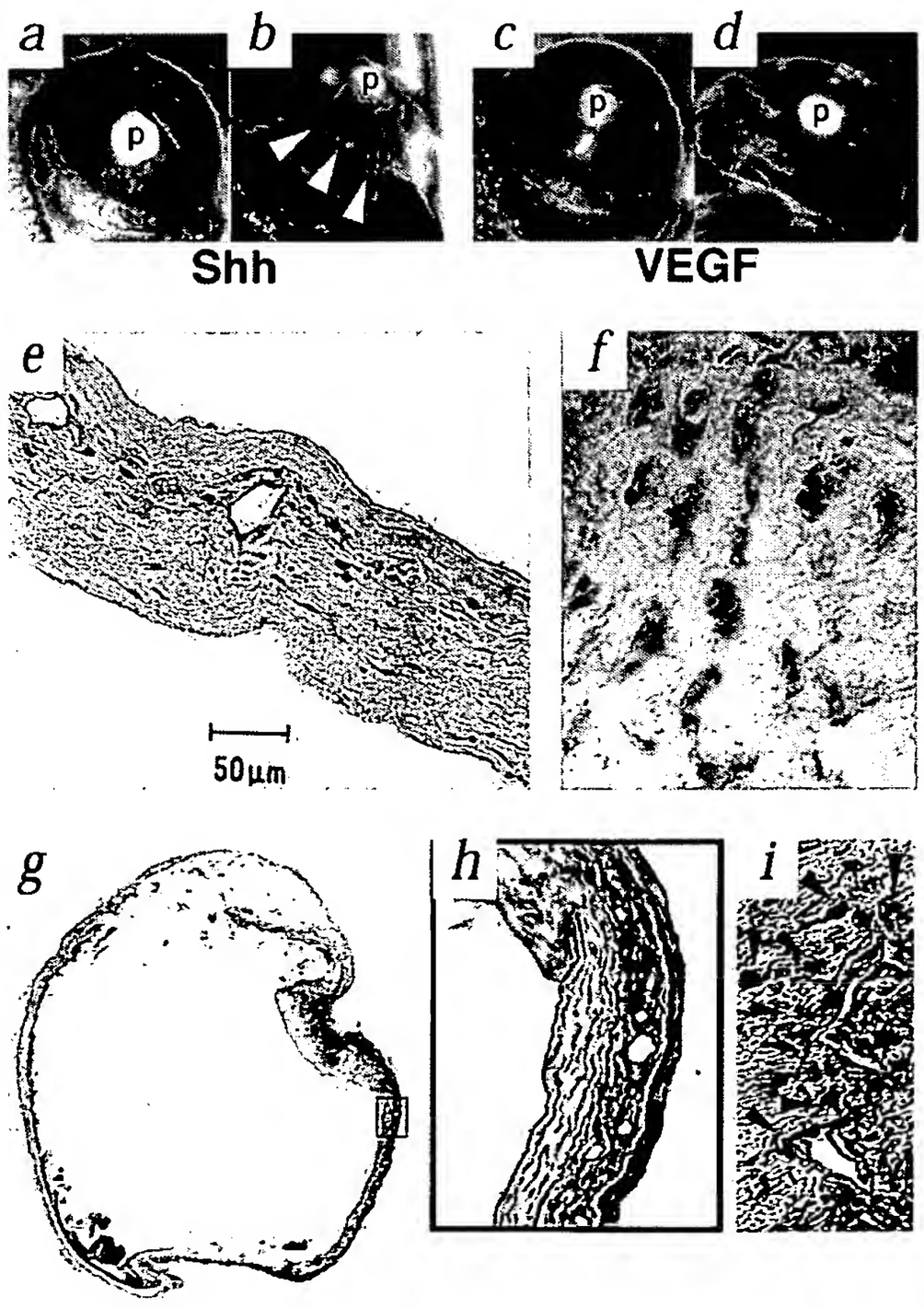


Fig. 3 Shh-induced angiogenesis has unusual morphological characteristics. ***a–c***, Neovascular growth is detectable in corneas implanted with pellets ('p') containing VEGF (*a*) and Shh (*b*), but not vehicle (*c*). ***d–h***, Shh (*d*, *g* and *h*), VEGF (*e*) and Shh+VEGF (*f*) induce vessels with different morphology. Red arrowheads indicate the main limbus artery, blue arrowheads indicate the main limbus vein, white arrowheads indicate expanded venous structures and the yellow arrowhead indicates an arteriovenous shunt. *h* shows branching vessels induced by Shh. ***i–k***, 5-μm cross sections of corneas treated with Shh (*i*), VEGF (*j*) or Shh+VEGF (*k*), immunostained for CD-31 (brown) show differences in vessel diameters induced by each treatment. ***l***, Vessel length, circumferential extent of neovascularity and average lumen diameter are significantly higher in Shh-treated corneas. When added to VEGF, Shh is able to increase average vascular lumen diameter (upper right); the large s.e.m. in Shh+VEGF-treated corneas reflects the presence of capillaries and large-diameter vessels. The number of vascular lumens per cross section is higher in VEGF-treated group. **, $P < 0.0001$; *, $P < 0.001$.

chemic hind-limb model, Shh-induced neovasculature consisted of large, branching vessels that grew directly from the limbus vessels and often extended to and surrounded the pellet at the apex of the new vessel growth (Fig. 3*b*, *d*, *g*, *h* and *i*). Many of these vessels exhibited dichotomous branching, creating a complex and well-organized vascular tree (Fig. 3*h*). The average number of branching vessels in corneal neovascularization induced by Shh was 7.3 ± 1.4 per field (data not shown). In contrast, VEGF implantation resulted in capillaries of lesser lumenal caliber that were uniformly distributed along the cornea (Fig. 3*a*, *e* and *j*). Shh-induced neovasculature also exhibited numerous large-diameter vessels that did not arise as branches of the limbus artery, but appeared to be venous structures that often formed arteriovenous shunts (Fig. 3*d* and *g*). The average length of Shh-induced neovessels was significantly greater than that of vessels induced by VEGF (1.05 ± 0.18 versus 0.67 ± 0.09 mm; $P < 0.0001$) (Fig. 3*l*). The circumferential extent of Shh-induced neovasculature was also increased compared with VEGF (190 ± 3.9 versus 116 ± 9.6 degrees; $P < 0.0001$) (Fig. 3*l*). Histological evaluation demonstrated increased lumenal diameters in Shh-induced versus VEGF-induced neovessels (32.62 ± 5.82 versus 7.25 ± 0.7 μm; $P < 0.0001$) (Fig. 3*i*, *j* and *l*). In both the Shh and VEGF groups, the number of periendothelial cells was limited with no significant difference (3.52 ± 1.66 versus 4.88 ± 1.75 smooth muscle cells per cross section, respectively; P = NS) (data not shown). In addition, the combination of Shh and VEGF showed lengthened, large-diameter neovessels like those seen with Shh alone, but also exhibited characteristics of VEGF-induced vasculature, that is, a dense area of fine vessels close to the implanted pellet (Fig. 3*f* and *k*). Thus, Shh and VEGF together appeared to produce an intermediate phenotype containing a variety of neovascular lengths and diameters (Fig. 3*f*, *k* and *l*).



Ptc1 mediates Shh-induced angiogenesis in fibroblasts

To determine the identity of cells directly activated by Shh during corneal angiogenesis, we implanted pellets containing Shh

Fig. 4 Shh acts upon stromal cells and induces VEGF production. ***a–d***, Macroscopic photographs of corneal neovascularization induced by pellets ('p') containing Shh (*a*) and VEGF (*c*) and correspondent β-gal staining for Ptc1 in Shh-treated (*b*) and VEGF-treated (*d*) corneas. β-gal–positive staining is detectable in correspondence of Shh-induced angiogenesis (arrowheads in *b*), but not of VEGF-induced angiogenesis (*d*). ***e–i***, Cross sections of Shh-treated corneas, prepared as in *b*, immunostained for CD-31 (*e*), vimentin (*f*) or VEGF at magnifications of ×20 (*g*), ×100 (*h*) and ×400 (*i*). VEGF staining is localized only in the neovascular area (*g*), around the neovessels (*h* and *i*). Cells with β-gal–positive nuclei have VEGF-positive cytoplasm (red arrowheads in *i*).

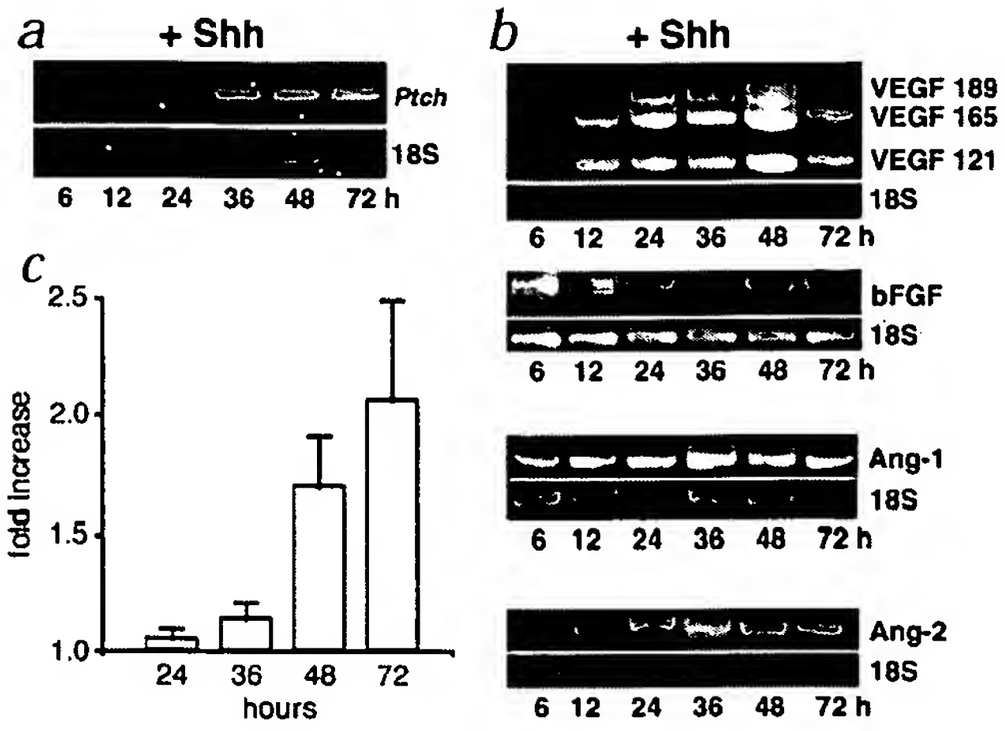


Fig. 5 Shh upregulates *Ptch*, VEGF and angiopoietins in human fibroblasts. ***a*** and ***b***, Quantitative RT-PCR for *Ptch* (*a*) and angiogenic cytokines (*b*) shows that fibroblasts respond to Shh by upregulating Ptc1, all 3 isoforms of VEGF-1, Ang-1 and -2, while bFGF is downregulated. ***c***, Conditioned media from Shh-stimulated compared with vehicle-stimulated human lung fibroblasts shows a ~2-fold increase in $VEGF_{165}$ (mean ± s.e.m.) detected by ELISA between 48 and 72 h in this representative experiment.

or VEGF into the cornea of NLS-*Ptch-lacZ* mice. After collecting them, we stained corneas for β-gal to detect Ptc1 expression. Strong β-gal staining was detected around the neovascular foci of NLS-*Ptch-lacZ* eyes treated with Shh, indicating that Shh activates the Hh pathway during neovascularization (Fig. 4*a* and *b*). In contrast, VEGF-treated corneas were β-gal–negative, indicating that VEGF does not induce expression of Ptc1 (Fig. 4*c* and *d*). Histological analysis showed that β-gal–positive cells were not endothelial cells (CD31-negative) or periendothelial cells (α-smooth-muscle-actin–negative, data not shown), but were consistent with interstitial fibroblasts (vimentin-positive) surrounding the neovessels (Fig. 4*e* and *f*). The β-gal–positive cells as well as their surrounding matrix were also immunopositive for VEGF, indicating that Shh might stimulate—either directly or indirectly—VEGF expression within the neovascular foci (Fig. 4*g*, *h* and *i*).

Shh upregulates *Ptch* and induces VEGF and Ang-1 and -2

We tested the possibility that Shh might induce fibroblasts to produce angiogenic cytokines by treating fibroblasts in culture with Shh protein and evaluating the induction of *Ptch*, VEGF and other angiogenic cytokines. Quantitative reverse transcriptase (RT)-PCR showed that a number of primary fibroblasts and fibroblast cell lines responded to Shh stimulation by upregulating *Ptch* (Fig. 5*a* and data not shown). Comparison of *Ptch* expression in Shh-treated and vehicle-treated fibroblasts at various time points showed that *Ptch* was induced within 6–12 hours after addition of Shh to the medium, and continued to increase up to 72 hours. In contrast, the absence of *Ptch* upregulation by endothelial cells in the corneal neovessels was mirrored *in vitro* by the inability of human umbilical vein endothelial cells (HUVECs) or microvascular endothelial cells to respond to Shh by *Ptch* upregulation, proliferation, migration or serum-free survival (data not shown).

In addition to upregulating *Ptch*, Shh stimulated cultured fibroblasts to increase expression of angiogenic growth factors, including all three isoforms of VEGF-1 and both Ang-1 and Ang-2 (Fig. 5*b*). Upregulation of mRNA encoding $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ was first detected at 12 hours and continued to increase up to 48 hours in Shh-stimulated compared with vehicle-stimulated cells at each time point. This increase in VEGF-1 mRNA correlated with a significant increase in $VEGF_{165}$ protein (Fig. 5*c*). Finally, Shh treatment also upregulated Ang-1 and Ang-2 mRNA (Fig. 5*b*). In contrast, expression of bFGF was decreased after Shh treatment (Fig. 5*b*). These results demonstrate that Shh induces a specific subset of angiogenic growth factors including the VEGF-1 isoforms as well as Ang-1 and Ang-2.

Discussion

Our results clearly show that Shh has angiogenic activity. Shh induces robust neovascularization in the setting of ischemia and may have important therapeutic utility in the treatment of ischemic disorders. Neovascularization induced by Shh appears to be mediated by stromal cells producing a combination of potent angiogenic factors, including VEGF, Ang-1 and Ang-2. *In vitro*, most fibroblasts cell lines respond to Shh by *Ptch* upregulation (Fig. 5*a* and data not shown). However, repeated attempts to activate HUVECs, aortic and microvascular endothelial cells by Shh treatment were unsuccessful. These cells show no proliferation, serum-free survival, migration or upregulation of Ptc1 in response to Shh proteins (data not shown). Ptc1 was not upregulated on endothelial cells in Shh-treated corneas or in endothelial cells of aortas from Shh-treated mice. Despite this, endothelial cells do express Ptc1 *in vitro* and *in vivo* and the possibility that Shh affects endothelial cells cannot therefore be completely excluded.

Our data instead indicate that neovascularization induced by Shh might be triggered through Shh/Ptc1 signaling specifically in mesenchymal cells. Fibroblasts are a well-known source of VEGF during development, tumor growth, hypoxia and inflammation[31–34]. Our data raise the possibility that VEGF production from fibroblasts might be mediated by the Hh pathway. Similar indirect mechanisms of inducing angiogenesis have been demonstrated for PDGF (platelet-derived growth factor) BB and TGF (tumor growth factor) β1 , both of which promote angiogenesis via upregulation of VEGF and basic fibroblast growth factor (bFGF)[35]. Given this precedent, we propose that Shh acts upon interstitial mesenchymal cells (such as fibroblasts in the cornea) to induce an array of angiogenic growth factors, including three isoforms of VEGF-1 as well as Ang-1 and Ang-2. The ability to upregulate these angiogenic cytokines in concert appears thus far unique to Shh. There are no previous reports of Ang-1 expression being regulated by other cytokines, morphogens, growth factors or ischemia.

Here we show that the angiogenic response to Shh is characterized by long, tortuous vessels with large diameters. It has been shown that vessels with increased length, diameter and branching are induced when Ang-1 acts synergistically with VEGF (ref. 36). We show here that Shh upregulates both VEGF and Ang-1; however, Shh induces an even more complex vascular system. When Shh is used, the quantitative and qualitative features of the vessels are more pronounced and they are also associated with vascular tortuosity. The basis for this remains to be elucidated, but it is possible that exogenous administration of VEGF and Ang-1 together might not be comparable to localized activation of these growth factors in stromal cells by Shh. Localized overexpression of VEGF and Ang-1 in the skin of transgenic mice, for example, produces

similar large-diameter, long and distinctly branching vessels[37]. Moreover, the sequence and magnitude of upregulation of these cytokines by Shh *in vivo* is unknown. Shh also upregulates Ang-2 and all three isoforms of VEGF-1. In colon cancer, compared with tumors expressing only one or two VEGF-1 isoforms, the coordinated expression of three VEGF-1 isoforms correlates with more aggressive tumors, as shown by vein invasion and metastasis leading to a poor prognosis[38]. The particular combination of angiogenic growth factors induced by Shh might thus contribute to the robust and distinct character of its neovascularization.

VEGF has been implicated in the earliest stage of vasculogenesis, during endothelial-cell differentiation and plexus formation, but also in postnatal angiogenesis through its ability to induce endothelial-cell migration and proliferation[39]. Ang-1 is required for both embryonic remodeling of the vascular plexus and postnatal vessel remodeling involving sprouting, branching or vessel maturation[39]. *In vivo* studies reveal that Ang-1 acts in a complementary and coordinated fashion with VEGF, mediating interactions between endothelial cells and surrounding support cells[40]. Ang-2 acts as a natural antagonist of Ang-1 (ref. 40). Whereas Ang-1 is expressed widely in normal adult tissues, Ang-2, in its role in continuous vascular stabilization, is highly expressed only at sites of vascular remodeling in order to allow the vessels to revert to a more plastic and unstable state[40]. Ang-2 is expressed along with VEGF in tumor vasculature and the two together might function as an angiogenic signal at the growing periphery of tumors[40]. Our study indicates that Shh upregulates both Ang-1 and Ang-2. The significance and relevance of this concomitant activation is unclear. We suggest that in the case of Shh-induced angiogenesis, VEGF might initiate the angiogenic response and angiopoietins could subsequently antagonize each other in a complex process of recruitment, stabilization and remodeling of neovasculature.

Shh-induced vessels tend to bifurcate into two branches that eventually split again. Previous reports show that tracheal splitting and branching during lung organogenesis are regulated by the Hh/Ptc1/Gli pathway through a number of effects including FGF inhibition[3]. We observed evidence of bFGF dowregulation in fibroblasts treated with Shh, and that the Shh-induced vessels are highly branched. The vascular network induced by Shh is also characterized by several venous structures with arterovenous shunts. This vasculature is functional, as demonstrated by the increase in perfusion and consequent rate of limb salvage in aged mice with limb ischemia. These experiments indicate that Shh might have therapeutic uses in promoting angiogenesis in the ischemic disorders.

The signaling pathway by which Hh upregulates these angiogenic growth factors remains to be determined. *Ptch* and many other Hh-inducible genes are regulated by the Hh pathway transcriptional factor Gli. However, no Gli response elements are present in the VEGF or Ang-1 promoter regions. Hh can, however, also induce a Gli-independent pathway that activates the orphan nuclear receptor, COUPTFII (ref. 41). Interestingly, COUPTFII-deficient embryos are defective in maturation of the primary vascular plexus and exhibit decreased Ang-1 expression[42]. Thus it is possible that Hh induces at least Ang-1 via COUPTFII activation in mesenchymal cells.

The development of functional vasculature requires precise spatial-temporal regulation of cell proliferation, migration, interaction and differentiation. The role of Shh as a morphogen might be relevant to its potential activity to orchestrate appropriate spatial-temporal production of angiogenic growth factors during embryonic and postnatal angiogenesis, which in addition must be coordinated with muscle, bone and nerve development. This report thus establishes novel biological and potentially therapeutic activities for Shh. The discovery of angiogenic activity for Shh, combined with its known morphogenic functions in development, indicates that Shh might coordinate epithelial/stromal interactions with the ingrowth of vasculature during development. Given that Shh can promote limb salvage in aged mice through the enhancement of blood flow and capillary density and induction of large caliber vessel formation, we suggest that Shh merits investigation as proangiogenic therapy for ischemic disorders.

Methods

Mice. Male C57BL/6J mice (Jackson Labs, Bar Harbor, Maine), heterozygous male or female NLS-*Ptch-lacZ* mice or their wild-type littermates (Ontogeny, Cambridge, Massachusetts) were used. All experiments were conducted in accordance with St. Elizabeth's or Biogen Institutional Animal Care and Use Committee.

Systemic treatment with Shh. Postnatal day 6 NLS-*Ptch-lacZ* mice were treated with daily subcutaneous injections of 10–20 µL of polyethylene glycol 20,000-conjugated C24II/A192C Shh N-terminal protein or vehicle[43]. Hearts and aortas were collected at postnatal day 9 and stained for β-gal expression.

Ischemic hind-limb model. Unilateral hind-limb ischemia was created in 2-year-old C57BL/6J mice[44]. Eighty mice were operated and treated with intramuscular injections of 1 mg/kg Shh–mIgG1 fusion protein, vehicle or 100 µg/kg of rhVEGF$_{165}$ (Chemicon, Temecula, California). Injections were once every other day during the first week, once every 3 days during the second week, and twice during the third and fourth weeks. At predetermined time points, necrosis and hind-limb perfusion were examined by two blinded operators[44]. Mice were then killed for histological analysis. Hind limbs were fixed in 100% methanol and cut in paraffin sections. Capillaries were counted by two blinded observers[44]. Shh–mIgG1 has increased half-life and activity *in vivo* (Shapiro *et al.*, manuscript in preparation). It contains residues Cys24–Gly197 of the human Shh coding sequence with two mutations: Cys24Ilelle and KRRH(32–35)QRRP, with a 16-fold increased activity *in vitro* compared with unmodified mature human Shh protein produced *E. Coli* (Taylor *et al.*, manuscript in preparation). The Fc region of mouse IgG1 was fused directly downstream of Gly127. The glycosylation site was destroyed with a Gln to Asn mutation. Protein was expressed in *Pichia pastoris* GS115 (Invitrogen, Carlsbad, California) using a pPIC9-derived vector and the α-mating-factor secretion signal. The protein was purified and sequenced as described[10,45].

Cornea neovascularization assay. Pellets containing one of the following were implanted in C57BL76J mice[36]: 1.5 µg myristoylated-Shh protein (Myr-Shh), 0.3 µg VEGF (R&D Systems, Minneapolis, Minnesota), 1.5 µg Myr-Shh + 0.3 µg VEGF, or vehicle. In NLS-*Ptch-lacZ* mice pellets contained Myr-Shh 1.5 µg/pellet, VEGF 0.3 µg/pellet or vehicle. Myr-Shh was prepared by chemical myristoylation (Taylor *et al.*, manuscript in preparation) of the α-amino group of Cys24 (of *E. Coli*-produced mature human Shh protein) followed by repurification and sequencing[10,45]. Myr-Shh exhibited 160-fold increased activity *in vitro* compared with mature human Shh protein (Cys24–Gly197).

Histology. Tissues from NLS-*Ptch-lacZ* mice were fixed in 0.2% gluteraldehyde, washed, stained overnight at 37 °C in 1 mg/mL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 50 mM Na_2HPO_4 pH8, and visualized as whole mounts or paraffin sections. For immunohistochemistry, eyes were fixed in 100% methanol or in 1% paraformaldehyde. Corneal hemispheres were cut into paraffin or frozen sections. Endothelial cells were identified using rat monoclonal antibody against mouse CD31 (Pharmingen, San Diego, California) and a biotinylated goat

immunoglobulin against rat. For periendothelial cells, a mouse monoclonal antibody against smooth muscle α-actin conjugated with alkaline-phosphatase (Sigma,) was used. For VEGF, a rabbit polyclonal antibody against VEGF (Santa Cruz Biotechnology, Santa Cruz, California) and a biotinylated goat immunoglobulin antibody against rabbit (Signet Labs, Dedham, Massachusetts) were used. Staining for vimentin was done with goat serum against vimentin (Sigma) compared with normal goat serum (Sigma) using horseradish peroxidase-conjugated donkey secondary antibody against goat (Jackson Immunoresearch, West Grove, Pennsylvania). For fluorescence microscopy, mice received an intravenous bolus of 500 µg of FITC-conjugated BS-1 lectin (Vector, Burlingame, California) 30 min before death. Eyes were fixed in 1% paraformaldehyde, and the dissected corneas were placed on glass slides.

Competitive RT-PCR. RNA was extracted from CCD37 human lung fibroblasts (ATCC) stimulated *in vitro* with MyrShh or vehicle. cDNA was obtained and amplified using the SuperScript preamplification system (Gibco-BRL, Paisley, UK). Signals were normalized to 18S rRNA using optimal 18S primer/Competimer ratios as determined for each target gene following the manufacturer's recommendations (Ambion, Austin, Texas) or to GAPDH, using GAPDH control reagents and Taqman analysis (PE Applied Biosystems, Foster City, California). The following primer pairs and PCR conditions were used. Ptc1: 5′-TCAGGATGCATTTGACAGTGACTGG-3′ and 5′- ACTCCGAGTCGGAGGAATCAGACCC-3′ with 25 cycles of 94 °C (30 s), 55 °C (1 min) and 72 °C (1 min). VEGF: 5′-CGAAGTGGTGAAGTTCATGGATG-3′ and 5′-TTCTGTATCAGTCTTTCCTGGTGAG-3′ with 30 cycles of 94 °C (30 s), 62 °C (1 min) and 72 °C (1 min). bFGF: 5′-TACAACTTCAAGCAGAAGAG-3′ and 5′-CAGCTCTTAGCAGACATTGG-3′ with 25 cycles of 94 °C (30 s), 62 °C (1 min), and 72 °C (1 min). Ang-1: 5′-CAACACAAACGCTCTGCAGAGAGA-3′ and 5′-CTCCAGTTGCTGCTTCTGAAGGAC-3′ with 25 cycles of 94 °C (30 s) and 64 °C (90 s). Ang-2: 5′-AGCGACGTGAGGATGGCAGCGTT-3′ and 5′-ATTTCCTGGTTGGCTGATGCTGCTT-3′ with 32 cycles of 94 °C (30 s) and 64 °C (90 s).

ELISA. $VEGF_{165}$ in conditioned media from MyrShh-stimulated cells were compared with vehicle-stimulated cells. $VEGF_{165}$ was measured per manufacturer's instructions using the Quantikine human VEGF-ELISA kit (R&D Systems, Minneapolis, Minnesota). All experiments were performed in triplicate.

Statistical analysis. All results are expressed as mean ± s.e.m. Differences were analyzed by ANOVA or χ-square test and considered statistically significant at $P < 0.05$.

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Postnatal Recapitulation of Embryonic Hedgehog Pathway in Response to Skeletal Muscle Ischemia

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Background—Hedgehog (Hh) proteins are morphogens regulating epithelial–mesenchymal signaling during several crucial processes of embryonic development, including muscle patterning. Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehog constitute the repertoire of Hh genes in humans. The activities of all 3 are transduced via the Patched (Ptc1) receptor. Recent observations indicate that exogenous administration of Shh induces angiogenesis. Here, we studied whether the endogenous Hh pathway, in addition to its functions during embryogenesis, plays a physiological role in muscle regeneration after ischemia in adults.

Methods and Results—We found that skeletal muscle ischemia induces strong local upregulation of Shh mRNA and protein. In addition, the Ptc1 receptor is activated in interstitial mesenchymal cells within the ischemic area, indicating that these cells respond to Shh and that the Shh pathway is functional. We also found that Shh-responding cells produce vascular endothelial growth factor under ischemic conditions and that systemic treatment with a Shh-blocking antibody inhibits the local angiogenic response and the upregulation of vascular endothelial growth factor.

Conclusions—Our study shows that the Hh signaling may be recapitulated postnatally in adult and fully differentiated muscular tissues and has a regulatory role on angiogenesis during muscle regeneration after ischemia. These findings demonstrate a novel biological activity for the Hh pathway with both fundamental and potential therapeutic implications. **(*Circulation*. 2003;108:479-485.)**

Key Words: genes, hedgehog ■ ischemia ■ muscle, skeletal ■ angiogenesis ■ tissue regeneration

In the past decade, there has been increasing appreciation of the fact that pathways studied predominantly during embryogenesis and known to be relatively silent during normal adult life may be recruited postnatally in response to tissue injury.[1]

Hedgehog (Hh) proteins are morphogens that act in a wide variety of tissues during embryonic development[2–9] and regulate epithelial–mesenchymal interactions that are crucial to morphogenesis of the nervous system, somite, limb, lung, gut, hair follicle, and bone.[3–7,10–12] There are 3 highly conserved Hh genes in mammals: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh).[13,0] The interaction of Hh proteins with their specific receptor patched-1 (Ptc1) inactivates the repression of the transmembrane protein smoothened (Smo), leading to activation of the transcription factor Gli,[14–16] the principal mediator of the Hh signaling pathway. Gli induces expression of downstream target genes of the Hh pathway, including Ptc1 and Gli itself.[17,18] Thus, Ptc1 and Gli are both components and transcriptional targets of the Hh signaling pathway.

Previous observations have suggested that Hh might also be involved in the vascularization of certain embryonic tissues. First, transgenic overexpression of Shh in the dorsal neural tube is associated with hypervascularization of neuroectoderm,[19] whereas a knockout of the zebra fish Shh homologue results in disorganization of the endothelial precursor cells and inability to form the dorsal aorta or axial vein. In addition, Shh-null mice lack proper vascularization of the developing lung.[4] More recently, it has been reported that vasculogenesis in the mouse embryo is regulated by Ihh.[20] Finally, very recently, we found that cells in the adult cardiovascular tissues express Ptc1 and can respond to exogenous administration of Shh by upregulating Ptc1.[21] We also demonstrated that Shh induces neovascularization in 2 different murine models of angiogenesis and upregulates 2 families of angiogenic growth factors, including vascular endothelial growth factor (VEGF) and angiopoietins.[21]

The aim of this study was to investigate whether the endogenous Hh pathway is physiologically involved in the

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Exhibit 3

revascularization of ischemic tissue in adults. We used a murine model of muscle regeneration by inducing ischemia of the hindlimb. Then, we observed the expression pattern of different components of the Hh pathway, including Shh, Dhh, Ihh, and Ptc1, and studied the relationship between Hh activation, VEGF expression, and angiogenesis. We found that Shh is activated in the regeneration after ischemia and that interstitial cells within the ischemic area strongly express Ptc1, indicating the postnatal activity of the Hh signaling pathway. We found that Ptc1 expression was associated with VEGF production and angiogenesis. Inhibition of Shh inhibits its endogenous angiogenesis and VEGF production in the ischemic hindlimb. Our data suggest a novel and unexpected physiological role for Shh.

Methods

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Me), male or female nls-Ptc1-lacZ mice, or their wild-type littermates (Ontogeny, Inc) were used for the ischemic hindlimb experiments.[21] All the experiments were conducted in accordance with the St Elizabeth's or Biogen Institutional Animal Care and Use Committee.

Murine Ischemic Hindlimb Model

Ischemia was induced in 8- to 12-week-old C57BL/6J mice, nls-Ptc1-lacZ mice, and their wild-type littermates as described previously.[21,22]

In Situ Hybridization for Hh Members

Skeletal muscles were harvested 4 and 7 days after surgery and immediately immersion-fixed overnight in 4% paraformaldehyde, paraffin-embedded, and sectioned longitudinally at 7 to 8 μm. Shh, Ihh, and Dhh in situ hybridization was performed with digoxigenin-labeled sense and antisense cRNA probes.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

Mice were killed 4 and 7 days after ischemia, and reverse transcription–polymerase chain reaction (RT-PCR) was performed as described previously. The primer sequences were as follows: Shh forward, GAGCAGACCGGCTGATGACT; Shh reverse, AGAGATGGCCAAGGCATTTAAC; Dhh forward, CGCAGACCGCCTGATGAC; Dhh reverse, GCGATGGCTAGAGCGTTCAC; Ihh forward, CAAACCGGCTGAGAGCTTTC; Ihh reverse, AGCCGACGCGGAGGAT. The probe sequences were as follows: Shh 6FAM, AGAGGTGCAAAGACA-MGBNFQ; Dhh 6FAM, AGCGTTGCAAAGAG-MGBNFQ; Ihh 6FAM, AGGTCATCGAGACTCA-MGBNFQ.

ELISA

Mice were killed 4 and 7 days after ischemia, and hindlimb muscle specimens were harvested and homogenized in lysis buffer. Shh protein levels were determined by ELISA as described previously.[23]

Western Blotting for Ptc1

Protein extracts from the skeletal muscles of mice killed 7 days after ischemia were used for Western blotting analysis of Ptc1 expression as described previously.[24] Densitometric analysis was performed (NIH Imaging program) to allow for quantitative comparison of protein expression.

Immunofluorescence and Immunohistochemistry

Ischemic and contralateral muscle specimens were harvested 7 days after induction of ischemia, and frozen sections were processed as detailed previously.[20,25] Primary antisera were goat polyclonal IgG anti-Shh C-terminus, goat polyclonal IgG anti-Ihh C-terminus, and goat polyclonal IgG anti-Dhh C-terminus (Santa Cruz Biotechnology). Horse anti-goat IgG horseradish peroxidase–conjugated antibody (1:500 dilution) (Vector Laboratories) was used as secondary antiserum. Staining was visualized by using FITC-conjugated streptavidin (Pharmingen). For vimentin immunostaining, muscles were fixed in 1% paraformaldehyde for 2 hours. The staining was done on frozen sections with anti-vimentin goat serum (Sigma) compared with normal goat serum (Sigma) using horseradish peroxidase–conjugated donkey anti-goat secondary antibody (Jackson Immunoresearch) or rhodamine-conjugated donkey anti-goat antibody (Santa Cruz Biotechnology). Staining for VEGF was performed with a rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology) with a biotinylated goat anti-rabbit Ig as secondary antibody.

LacZ Immunofluorescence and Histochemistry in nls-Ptc1-lacZ Mice

For β-gal immunofluorescence staining, hindlimb muscles from nls-Ptc1-lacZ mice were harvested and processed as described previously.[26]

Inhibition of the Hh Pathway and Analysis of Local VEGF Expression and Analysis of the Angiogenic Response to Ischemia

Unilateral hindlimb ischemia was induced as described above in 8- to 12-week-old C57BL/6J mice. Animals were treated with daily intraperitoneal injections of 10 mg/kg 5E1 blocking antibody or the same amount of 1E6 control antibody. 5E1 blocks the binding of Shh to Ptc1; it was obtained from Curis Inc and Dr Thomas Jessell (Columbia University) and prepared as purified IgG1 in PBS.[18] Seven days after induction of ischemia, mice were killed. Hindlimb muscle specimens were harvested, processed, and analyzed by Western blotting for VEGF expression. For analysis of the response to ischemia, animals were divided into 2 groups: the first group received a total dose of 1.25 mg 5E1, delivered systemically via an osmotic pump over a period of 21 days, and the second group received an equal amount of 1E6. Ten animals in each group were studied. At days 7, 14, 21, and 28 after induction of ischemia, blood flow was measured with a laser Doppler perfusion imaging system as described previously.[20,21]

Analysis of capillary density was performed as described previously.[21,22]

Statistical Analysis

All results are expressed as mean±SD, with the exception of the real-time RT-PCR results, which are presented as mean±SEM. Group differences were analyzed by ANOVA or Student's t test. Differences were considered statistically significant at a value of $P<0.05$.

Results

Shh Signaling Pathway Is Activated in Ischemic Regenerating Skeletal Muscles

The expression of Shh mRNA was increased at 4 and 7 days after injury in ischemic compared with nonischemic skeletal muscle, as detected by in situ hybridization (Figure 1a). Shh mRNA in ischemic muscle was strongly induced, particularly in the interstitial regions. Expression of Ihh was also slightly elevated in ischemic muscle, whereas little Dhh expression was detected at 4 or 7 days after ischemic injury (Figure 1b).

Upregulation of Shh was confirmed and quantified by real-time RT-PCR: Shh expression increased 10±3.3-fold in day 4 ischemic muscles ($P=0.01$) and 16±3.8-fold at day 7 ($P=0.0007$) (Figure 2a and data not shown). No significant increment of Dhh and Ihh expression was documented.

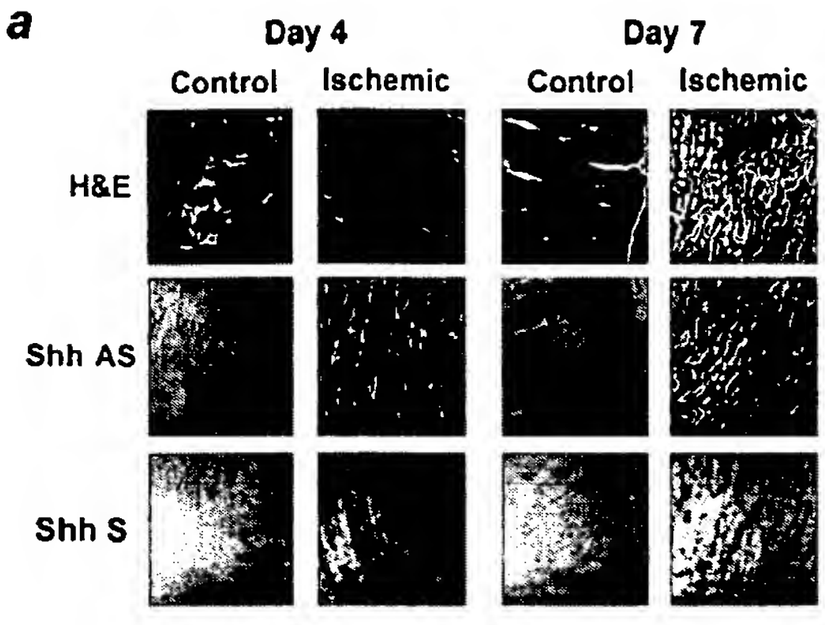

Figure 1. Shh mRNA is upregulated during skeletal muscle regeneration after ischemia. In situ hybridization for Shh, Ihh, and Dhh 4 and 7 days after ischemia. Strong positive signal for Shh mRNA is present in interstitium of ischemic muscles at both days 4 and 7 after surgery, whereas no signal is detected in contralateral nonischemic specimens (a); Ihh mRNA seems slightly elevated in ischemic muscle (b). Very little Dhh expression is detected at 4 or 7 days after ischemic injury (b) (H&E indicates hematoxylin and eosin; Shh AS, Shh Antisense probe; Shh S, Shh Sense probe; Ihh AS, Ihh Antisense probe; Dhh AS, Dhh Antisense probe).

The concentration of Shh protein in ischemic and contralateral muscles was studied by ELISA and was shown to be increased at day 4 (P=0.03) and day 7 (P=0.02) (Figure 2b). Ptc1 upregulation was verified by Western blotting (Figure 2c) and normalized for tubulin expression, was also statistically significant (P<0.01) (Figure 2d).

Immunofluorescence analysis demonstrated that several cells are immunopositive for Shh within 7 days after induction of ischemia (Figure 3, a and b). No positive immunostaining in ischemic muscle was observed at this time point for either Ihh or Dhh (data not shown). Immunofluorescence staining for β-gal, performed in nls-Ptc1-lacZ mice, demonstrated the expression of Ptc1 in several cells in the ischemic tissue (Figure 3, c and d). Interestingly, both Shh- and Ptc1-positive cells appeared to be interstitial cells widely distributed in the ischemic area, within and around skeletal muscle fibers. By performing double immunofluorescence staining for Shh and β-gal in nls-Ptc1-lacZ mice, we demonstrated that Shh and Ptc1 are coexpressed in the same cells (Figure 3, e–g). These data demonstrate that cells in the ischemic tissue produce Shh and express the Ptc1 gene, indicating that the Shh pathway is physiologically active during muscle regeneration after ischemia in adults. Because Ptc1 is a downstream transcriptional target of the Shh signal transduction pathway and Ptc1 expression is known to occur in response to Shh signaling, expression of Ptc1 constitutes evidence of active Shh signaling in ischemic skeletal muscle. In addition, these findings suggest that in this model, an autocrine interaction occurs between the Shh ligand and its receptor Ptc1.

Shh and Ptc1 Activation Occur in Interstitial Mesenchymal Cells and Are Associated With VEGF Production

To determine the identity of the Shh-producing and -responding cells during ischemia, we performed further immunohistochemical analyses. These interstitial cells were not positive for the endothelial cell marker CD31 or the smooth muscle cell marker α-SM-actin (data not shown). In contrast, we found that Shh- and Ptc1-positive cells stained positive for vimentin, consistent with mesenchyme-derived fibroblasts (Figure 4, a–d).

We also analyzed the relationship between the activated, endogenous Shh pathway and VEGF in ischemic skeletal muscle. We found that Ptc1-positive interstitial cells located within the ischemic area were strikingly immunopositive for VEGF (Figure 4e). The colocalization in the same cells of Ptc1 and VEGF suggests that the Shh signaling pathway may stimulate, either directly or indirectly, VEGF expression within the neovascular foci. No Ptc1 (X-gal) or VEGF staining was observed in either the contralateral or control muscles (data not shown).

The time course of upregulated Ptc1 expression after the onset of ischemia, inferred from whole-mount X-gal staining of nls-Ptc1-lacZ hindlimbs, was characterized by Ptc1 upregulation beginning 4 days after ischemia, peaking at day 7, and decreasing significantly by day 14 after ischemia (Figure 4f).

Inhibition of Shh Signaling Pathway Impairs Local VEGF Upregulation

To determine whether the Shh signaling pathway is necessary for VEGF upregulation during ischemia, we used a Shh-neutralizing antibody (5E1). After unilateral hindlimb ischemia had been induced in mice, animals were treated for 7 days with systemic injections of 5E1 or control antibody (1E6). Local VEGF expression was studied by Western blotting in both ischemic and contralateral muscles. As expected, mice treated with the 1E6 control antibody exhibited a physiological upregulation of VEGF in the ischemic hindlimbs (Figure 5a). In contrast, animals treated with the 5E1 antibody did not upregulate VEGF in the ischemic hindlimb (Figure 5a). Comparison of VEGF expression in ischemic muscle, normalized for tubulin expression, indicated a statistically significant difference between mice treated with 5E1 versus 1E6 (P<0.01) (Figure 5b).

Inhibition of the Shh Signaling Pathway Decreases the Angiogenic Response to Ischemia

Hh-blocking antibody (5E1) or a control antibody (1E6) was administered by continuous subcutaneous infusion through an osmotic pump for 3 weeks after induction of hindlimb ischemia. Twenty-eight days after induction of ischemia, blood flow was significantly lower in animals treated with the Shh-blocking antibody (P<0.01) (Figure 5c). Capillary den-

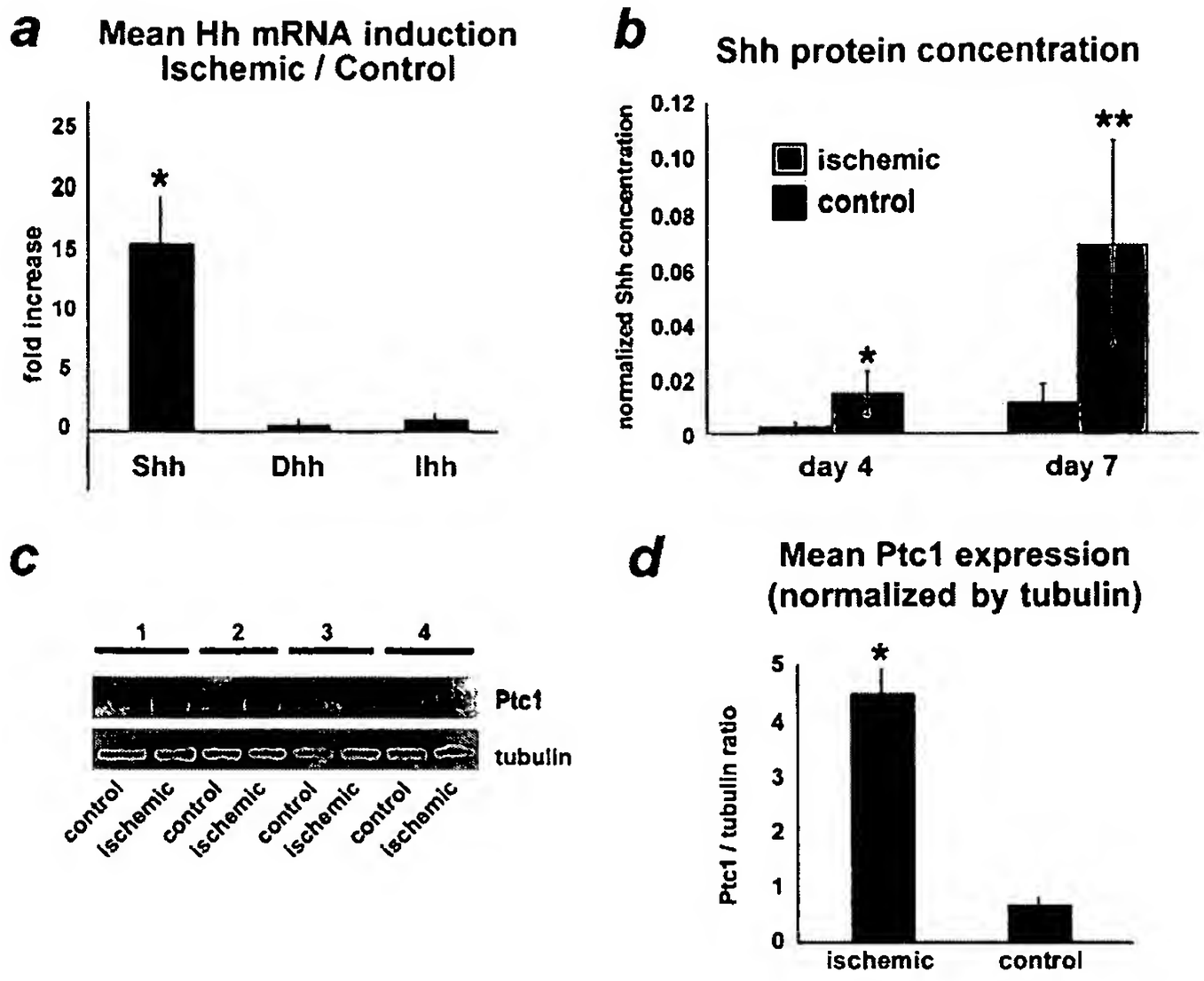

Figure 2. Quantification of Shh and Ptc1 upregulation during skeletal muscle regeneration after ischemia. Real-time RT-PCR for Shh, Ihh, and Dhh 7 days after ischemia (a): Shh mRNA is increased 16±3.8-fold (**P*=0.0007), whereas Dhh and Ihh gene expression are not substantially altered. ELISA for Shh 4 and 7 days after ischemia (b): Shh protein concentration, normalized by total protein concentration, is significantly increased in ischemic skeletal muscles at both time points (**P*=0.03 and ***P*=0.02). Representative Western blotting for Ptc1, 7 days after ischemia (c): Ptc1 signal is increased in ischemic muscles compared with controls. Quantification of Ptc1 upregulation (d): Ptc1 signal, quantified by densitometric analysis and normalized for tubulin, is upregulated significantly in ischemic tissues (**P*<0.01).

sity was assessed by CD31 immunostaining and was significantly reduced as well ($P<0.0001$) (Figure 5, d and e). These results indicate that the activation of the Shh pathway is a prerequisite for the postnatal angiogenic response to skeletal muscle ischemia.

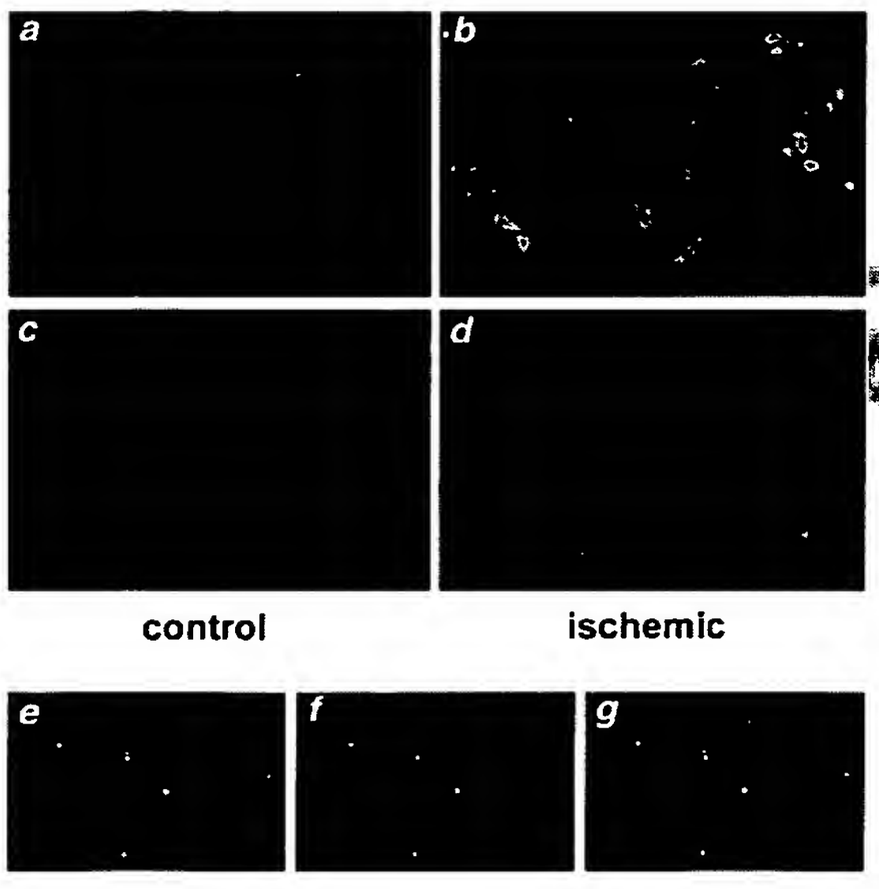

Figure 3. Immunostaining for Shh and Ptc1 in ischemic regenerating skeletal muscle. Cross sections of day 7 ischemic and nonischemic hindlimbs (magnification ×20). Immunofluorescence staining shows that Shh is strongly upregulated in several interstitial cells in ischemic tissues (a and b). Likewise, β-gal immunofluorescence staining in nls-Ptc1-lacZ mice (magnification ×20) demonstrates strong upregulation of Ptc1 during ischemia (c and d). Double immunofluorescence in nls-Ptc1-lacZ mice (magnification ×20) shows that Shh staining (e) and Ptc1 (β-gal) staining (f) are coexpressed in same interstitial cells (g).

Discussion

The Hh pathway has been studied and characterized extensively during embryogenesis. The vast majority of these prenatal studies have focused on the role of Hh family members in the regulation of epithelial–mesenchymal interactions crucial to limb, lung, gut, hair follicle, and bone formation,[3–6] including a possible role during vascularization of certain embryonic tissues.[4,19,20,27–29] In contrast, a role for the Hh family in the regulation of postnatal tissue regeneration and revascularization has received limited attention.[30–32] We recently demonstrated that exogenous administration of Shh induces neovascularization in both corneal and ischemic hindlimb models of angiogenesis.[21] Shh stimulates fibroblasts in vitro to produce a combination of potent angiogenic factors, including the 3 major isoforms of VEGF, Ang-1, and Ang-2.[21] Shh seems to act as an indirect angiogenic agent and may trigger neovascularization through Shh/Ptc1 signaling specifically in mesenchymal cells.[21]

Following these observations, we investigated the hypothesis that the Hh pathway may be postnatally recapitulated in response to skeletal muscle ischemia and discovered that in adult mice, a strong upregulation of Shh and Ptc1 occurs during regeneration of ischemic skeletal muscle. These findings are consistent with previous reports in the literature describing the association of both ischemia and tissue regeneration with the reactivation of genes involved in fetal transcription programs.[33–35]

After ischemia, Ptc1 expression occurs in interstitial mesenchymal fibroblasts. The ability of fibroblasts to respond to Shh stimulation has already been demonstrated: eg, fibroblasts respond to Shh stimulation in vitro,[21] physiologically express Ptc1 in adult perineural sheaths and dermis,[6,9] and upregulate Ptc1 and VEGF during Shh-induced corneal neo-

vascularization.[21] Taken together, these data strongly suggest that fibroblasts are central mediators of Shh activity during muscle regeneration.

In our ischemic model, interstitial mesenchymal fibroblasts also expressed Shh. The coexpression of Shh and Ptc1 in the same cells indicates the presence of an autocrine mechanism regulating Shh signaling in ischemic muscle. Such an autocrine mechanism has already been described in adult pancreas, in which Dhh and Ihh are coexpressed with Ptc1 in pancreatic β-cells and regulate insulin production.[25]

In muscle regeneration after ischemia, a crucial role is played by angiogenesis.[22] In this study, we show that Ptc1-positive interstitial fibroblasts within the ischemic area produce VEGF. We also show that the inhibition of Shh signaling is sufficient to decrease local VEGF upregulation. Similarly, ischemia-induced angiogenesis is decreased by inhibition of the Shh pathway. These results indicate that although Ptc1-positive fibroblasts do not represent the majority of VEGF-producing cells during ischemia, the activation of the Shh signaling pathway is crucial for the overall production of VEGF and the related angiogenic response. Indeed, interstitial fibroblasts are important supporting cells, and their function, modulated by Shh, might be fundamental for stimulating the angiogenic activity of neighboring cells.

The mechanism by which ischemia and/or hypoxia upregulates Shh expression remains uncertain. The promoter regions

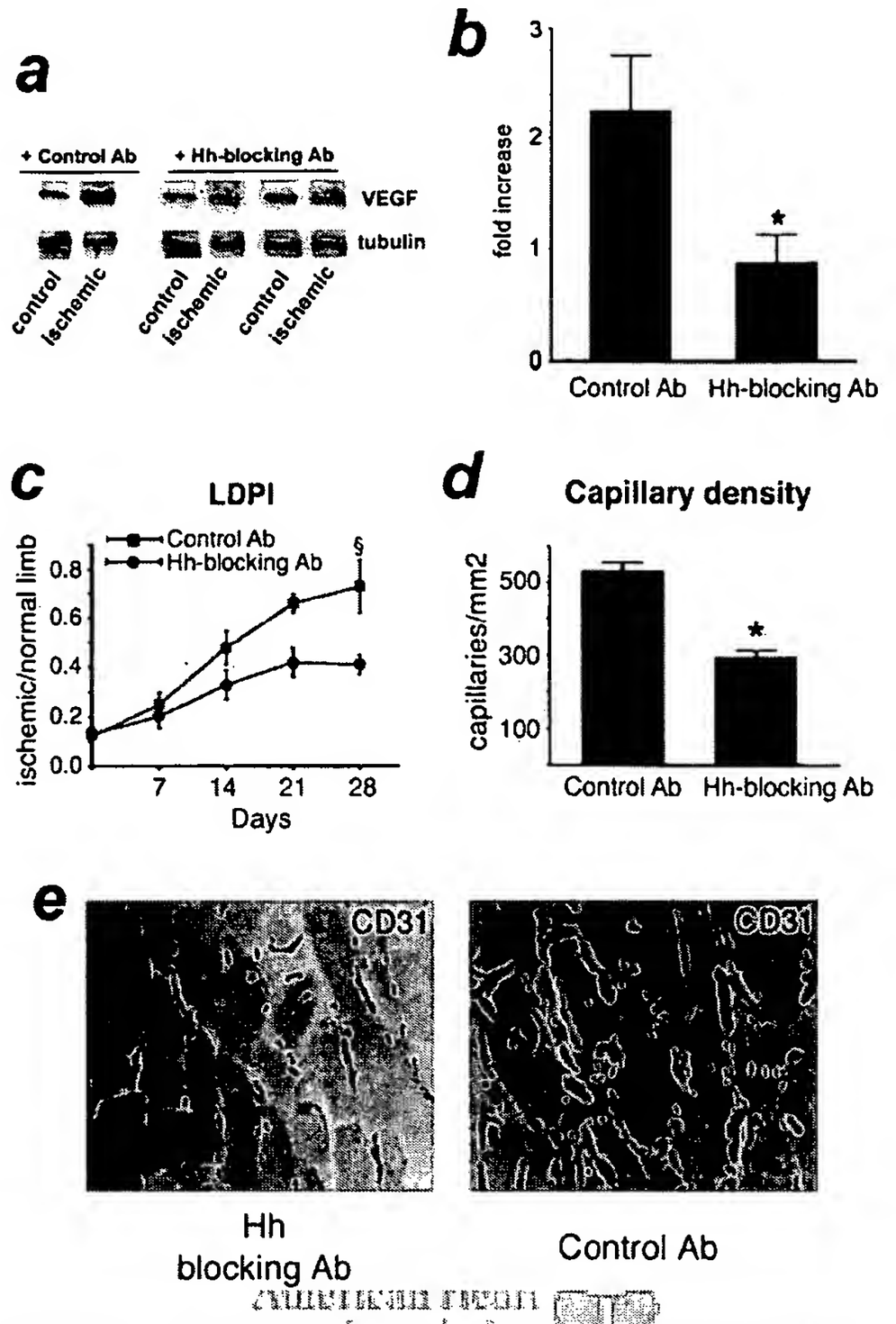

Figure 5. Inhibition of Shh signaling decreases VEGF upregulation and angiogenic response to ischemia. Hindlimb ischemia was induced in young mice, after which they were treated with a Shh-blocking antibody (Ab) (5E1) or a control antibody (1E6). Western Blotting analysis of VEGF expression shows upregulation in ischemic limbs of mice treated with 1E6 compared with normal limbs (a). No upregulation of VEGF was detected in ischemic limbs of mice in which Shh signaling was blocked by 5E1 (a). Quantification of VEGF signal normalized for tubulin in mice treated with 5E1 and 1E6 shows a statistically significant difference between 2 groups (*$P<0.01$) (b). Blood flow was assessed by laser Doppler perfusion imaging (LDPI) (c): no significant increase in hindlimb perfusion was seen over time in group treated with 5E1. In these animals, at day 28 after surgery, blood flow was reduced significantly compared with controls (0.414 ± 0.019 vs 0.741 ± 0.126, §$P<0.01$). Capillary density assessed 28 days after surgery was reduced significantly in mice treated with Shh-blocking antibody vs controls (*$P<0.0001$) (d). CD31 staining of skeletal muscle sections from ischemic hindlimbs of mice treated with 5E1 and control antibody (magnification ×10) (e): number of vessels is strikingly reduced in 5E1-treated tissues.

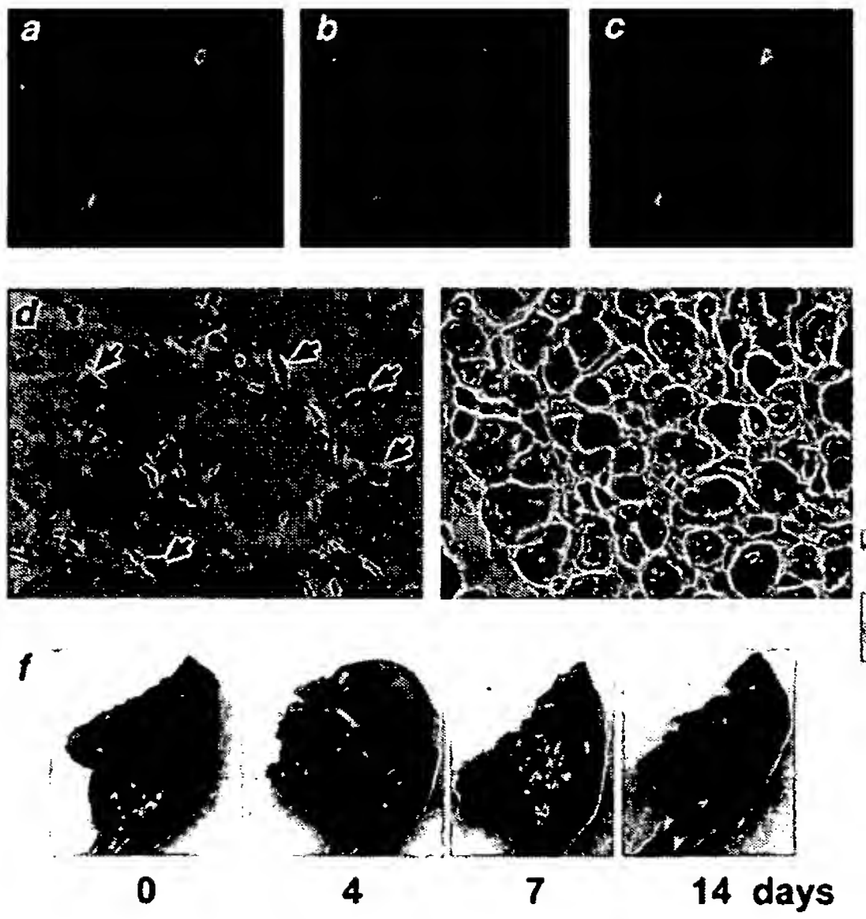

Figure 4. Ptc1 upregulation occurs in interstitial mesenchymal cells, is associated with VEGF production, and is maximal 7 days after ischemia. Shh and vimentin double immunofluorescence in skeletal muscle 7 days after ischemia: Shh staining (a) and vimentin staining (b) are colocalized in same cells (c) (magnification ×40). Staining for X-gal and vimentin in day 7 ischemic hindlimb of nls-Ptc1-lacZ mouse (magnification ×10) (d): Ptc1-positive cells (green nuclei) are interstitial mesenchymal cells (brown cytoplasm) (arrows). Staining for X-gal and VEGF in day 7 ischemic hindlimb of nls-Ptc1-lacZ mouse (magnification ×10) (e): Ptc1-positive mesenchymal cells (green nuclei) are VEGF-positive (brown cytoplasm) (arrows). Time course for Ptc1 expression after ischemia (f): whole-mount X-gal staining of ischemic hindlimbs from nls-Ptc1-lacZ mice shows that expression of Ptc1 increases progressively after ischemia, reaching its maximal upregulation at day 7.

of Hh family members are not known to include a hypoxia-inducible factor sequence, and no data are available about the possible interactions between Shh- and hypoxia-inducible factor pathways in regulating VEGF synthesis and stabilization. Interestingly, it has been reported that in mice with a deletion of the hypoxia-response element in the VEGF promoter, fibroblasts are still able to upregulate VEGF under hypoxic conditions.[36] This finding is apparently specific for fibroblasts and, in association with the ability of these cells to produce VEGF on Shh stimulation, indicates that fibroblasts

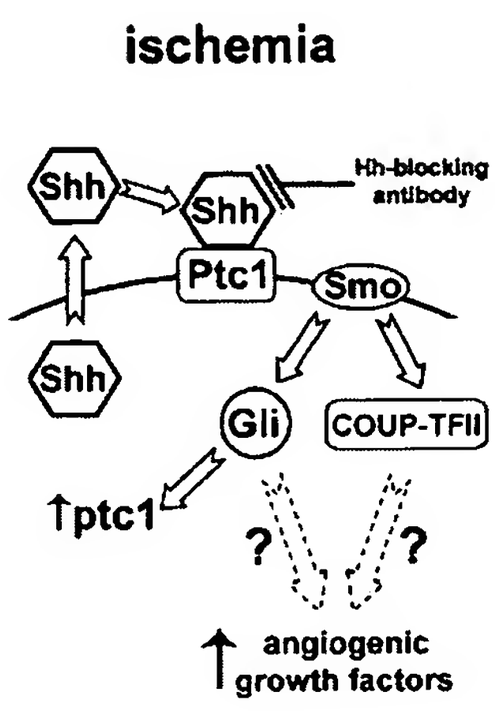

Figure 6. Schematic of proposed Shh-dependent signaling pathway responsible for upregulation of angiogenic growth factors during ischemia. Under ischemic conditions, Shh, produced by mesenchymal fibroblasts, binds receptor Ptc1 on membrane of Shh-producing cells. Interaction between Shh and Ptc1 results in activation of transmembrane protein Smo, which activates transcription factor Gli, responsible for upregulation of ptc1 gene. Alternatively, Shh may lead to activation of nuclear orphan receptor COUP-TFII through a Gli-independent pathway. Upregulation of genes encoding for angiogenic growth factors might depend on either Gli or COUP-TFII activation (open arrows). Shh pathway may be inhibited by an Hh antibody, which blocks binding of Shh to Ptc1.

may have hypoxia-independent mechanisms to upregulate VEGF, potentially involving direct regulation by the Hh pathway transcriptional factor Gli. However, no Gli response elements are present in the VEGF promoter region. Hh can, however, also induce a Gli-independent pathway, which activates the orphan nuclear receptor COUPTF-II.[37] Interestingly, COUPTF-II–null embryos are defective in maturation of the primary vascular plexus.[38] Thus, it is possible that the induction of angiogenic growth factors by Hh occurs via COUPTF-II activation in mesenchymal cells (Figure 6).

The development of functional neovasculature in regenerating tissues requires precise spatial–temporal regulation of cell proliferation, migration, interaction, and differentiation. The role of Shh as a morphogen may be relevant to its potential activity to orchestrate appropriate postnatal angiogenesis after tissue injury. The activation of components of the Hh pathway during ischemia and the reduced angiogenesis observed after inhibition of Hh suggest a crucial role for these morphogens in the pathophysiology of muscle regeneration. In addition, these results open the possibility that members of the Hh family might play a role in the development of angiogenesis-related diseases, such as diabetic retinopathy or tumor angiogenesis. Finally, influencing angiogenesis by modulating the Hh pathway might have important implications for both proangiogenic and antiangiogenic therapeutic strategies.

Acknowledgments

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